Title The effects of cytokines in a cartilage explant model system

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THE EFFECTS OF CYTOKINES IN A CARTILAGE EXPLANT MODEL SYSTEM

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A thesis submmited to the Faculty of Science, Technology and Design, University of Luton, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Abstract

Arthritis is a pathological condition whereby a persistent inflammatory response leads to breakdown of articular cartilage in synovial joints. Cartilage is a specialised avascular tissue containing chondrocytes embedded in an extracellular matrix. The cartilage matrix is composed of collagen to provide strength with aggregated proteoglycan to facillitate hydration. Cartilage has been reported to loose proteoglycans with concordant loss of integrity observed in arthritic disease pathology. Proteoglycans loss from cartilage has also been reported in *in vitro* models. Application of interleukin-1 (IL-1β) to cartilage *in vitro* has been demonstrated to increase loss of proteoglycans and modulate production of inflammatory mediators such as Nitric Oxide (NO) and Prostaglandin-E_2 (PGE_2). NO and PGE_2 have also been associated with cartilage breakdown. Other cytokines such as colony stimulating factors (CSFs) may regulate cartilage function. The aim of this study was to select a cartilage explant system and compare the effects of interleukin-1 (IL-1) with those of colony stimulating factors (CSFs) by measuring the production of NO and PGE_2 and release of proteoglycans.

It was found that IL-1β increased PGE_2 and NO production, but not loss of proteoglycans from rat cartilage explants. Granulocyte-CSF (G-CSF) and IL-3 increased production of NO and PGE_2, respectively. When combined, IL-1β / Granulocyte-Macrophage (GM-CSF) increased production of PGE_2 and G-CSF / IL-1β produced increased proteoglycan loss from explants.

The model was then modified by integrating Swiss 3T3 Fibroblasts monolayers with explants. Fibroblasts were initially screened to determine their separate response to these cytokines. Fibroblasts did not release proteoglycans into the culture media, but produced elevated concentrations of NO and PGE_2 in response to IL-1β.

Fibroblast-cartilage co-cultures treated with IL-1β produced increased NO, PGE_2 and proteoglycan release. G-CSF, GM-CSF and IL-3 caused increased levels of PGE_2 in co-cultures, however, IL-1β was required to generate significant proteoglycan loss from cartilage explants. Finally, extra-cellular signal related protein kinases 1 and 2 (ERK 1&2) and p38 intracellular signalling pathways were shown to be involved in IL-1β mediated production of NO fibroblasts and explants.

These studies show that IL-1β has increased potential to mediate cartilage breakdown when interacting with other cytokines, such as G-CSF, and other cell types, such as Swiss 3T3 fibroblasts. IL-1β has defined intracellular signalling pathways that may produce a range of responses in cartilage explants and fibroblasts. These studies may relate to production of inflammatory processes and loss of cartilage integrity and function in pathological conditions.

Authors Declaration

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of Luton. It has not been submitted before for any degree or examination in any other university.

Name: S. Ogden Date: 17/01/01

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List of Abbreviations

Abbreviation	Full Term
AA	Arachidonic acid
C/EBP	C/CAAT-enhancer binding protein
COX	Cyclooxygenase
CRP	Complement regulatory protein
CSF	Colony stimulating factor
CSM	Complete supplemented medium
DDA	Dimethyl dioctadecyl ammonium bromide
DMB	1,9 dimethyl methylene blue
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulphoxide
ECM	Extracellular matrix
E.Coli	*Escherichia coli*
EDTA	Ethylenediaminetetra-acetic acid
EGF	Endothelial growth factor
ERK	Extra-cellular signal related protein kinase
FCS	Fetal calf serum
FHC	Femoral head cartilage
GAG	Glycosaminoglycan
G	Granulocyte
GM	Granulocyte Macrophage

HA	Hyaluronic acid
HABR	Hyaluronic acid binding region
HBSS	Hank's balanced salt solution
IMS	Industrial methylated spirits
L-NAME	L-nitroarginine-methyl-ester
L-NMMA	N^G-mono-methyl-arginine
LPS	Lipopolysaccharide
MAP	Mitogen activated protein
MMP	Matrix metalloproteinase
NED	N-(1-Naphthyl)ethylenediamine
NF-κB	Nuclear factor kappa-B
NSAIDs	Non Steroidal anti-inflammatory drugs
NO	Nitric oxide
NOS	Nitric oxide synthetase
OA	Osteoarthritis
PG	Proteoglycan
PGE_2	ProstaglandinE_2
RA	Rheumatoid Arthritis
ROS	Reactive Oxygen Species
SA	Sulphanilimide
SNAP	S-nitroso-N-acetyl-penicillamine
STAT	Signal transducer and activator of transcription
TIMP	Tissue inhibitor of matrix metalloproteinase
TNFα	Tumour necrosis factor-α

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Finally, thanks to all my friends and family.

I dedicate this thesis to the memory of Jose Morris

Chapter1:

Introduction

1.1.0: Inflammation and arthritis

Inflammation is a normal physiological response that may be induced by tissue damage or foreign tissue recognition by the immune system. Inflammation is associated with diseases such as rheumatoid (RA) and osteoarthritis (OA). The purpose of the inflammatory response is to repair tissue damage, prevent further infection and remove any foreign material from an affected area. However, the inflammatory reponse is peristent within arthritic disease pathology and leads to connective tissue damage. The mechanisms involved in the progression of RA are distinct from those in OA. RA is a disease that principally involves damage to joints but also produces extra-articular manifestations. RA affects 0.03% to 1.5% of the population, with females affected 2 to 3 times more often than males. Life-span is decreased on average by 7.5 years for men and 3.5 years for women. Osteoarthritis (OA) is the most common joint disease and is strongly related to age. OA is a condition of synovial joints characterised by focal cartilage loss and an accompanying reparative bone response. Typical radiographic features are joint space narrowing and the presence of osteophytes and sclerosis. OA is an uncommon disease within individuals under 45 years, however, at least half the population have radiographic evidence of OA at 65 years. There are a number of similarities in the pathology of both RA and OA. A number of inflammatory mediators have been identified in both RA and OA that are associated with the destruction of articular cartilage and bone. It is these factors that may contribute to the changes from normal joint physiology to arthritic disease pathology.

1.1.1: Normal joint physiology

A normal joint is designed to carry out a wide range of movements. Synovial joints contain cartilage that is smooth in appearance and has shock absorbing properties (see Fig: 1.11.) required for normal movement. The joint is lined by membrane called synovium that consists of cells called synoviocytes. Synoviocytes have been categorised by Freemont, (1996) into two cell types; macrophage-derived phagocytes and mesenchymal connective tissue lineage derived cells that produce synovial fluid factors that aid joint lubrication. However, the nornal synovial joint physiology may undergo a series of changes that lead to disease pathology associated with arthritis disease pathologies.

Fig: 1.1.1: Diagram of Normal joint (A) and photograph of normal femoral head (B).

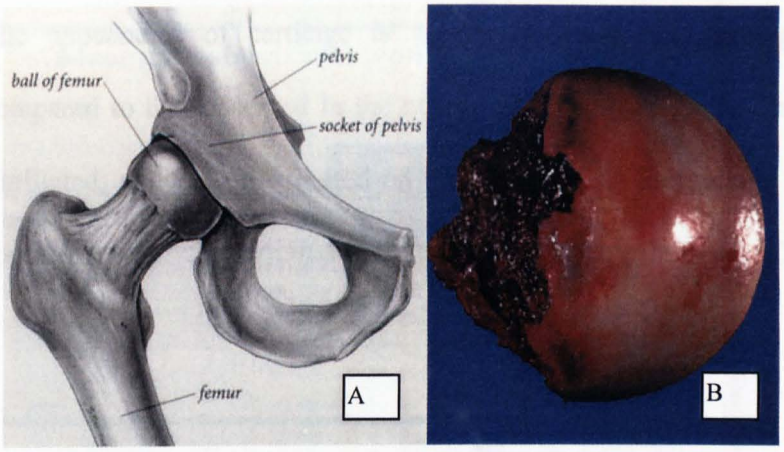


Fig: 1.1.1: A: Diagram shows the femoral head inter-locking into the pelvis to form a normal femoral joint (Picture adapted from American Academy of Orthopaedic Surgeons web page, see Appendix IV). B: Photograph shows the smooth appearance of cartilage coating the femoral head from a joint with normal physiology.

1.1.2: Disease joint pathology.

The joint space is infiltrated by a range of inflammatory cells such as T cells, macrophages and plasma cells in RA disease pathology. Cellular accumulation is accompanied by release of inflammatory mediators such as cytokines, chemokines reactive oxidative species (ROS), metalloproteinases and prostaglandins that promote the inflammatory process (Odeh, 1997). An increase in the number of cells comprising the synovium is also apparent in the pathological joint (Feldmann *et al.*, 1996). Intra-articular foreign material accumulates in synovium and

synovial fluid, consisting of breakdown products from articular cartilage, bone and the synovium (Freemont, 1996).

The appearance of cartilage is notably different in pathological conditions compared to that observed in the normal physiological joint. Cartilage surface is fibrillated, unevenly distributed on the joint surface and unable to confer shock absorbing properties required for normal movement, (see Fig: 1.1.2).

Fig: 1.1.2: Diagram of pathological joint (A) and photograph of a pathological femoral head (B).

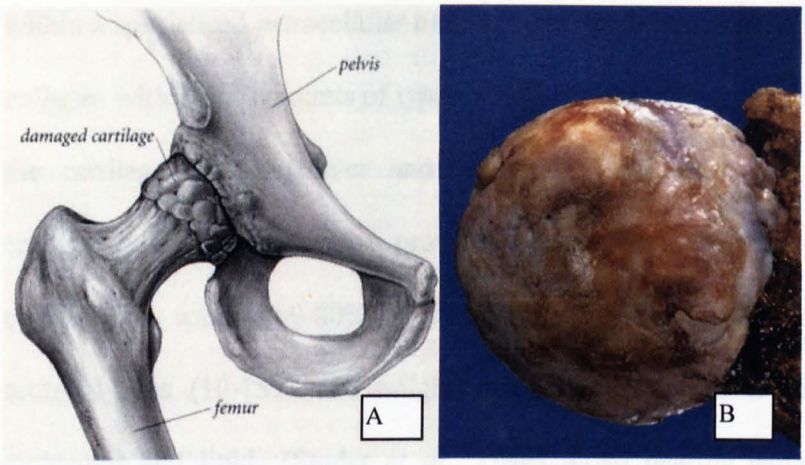


Fig: 1.1.2: A: Diagram shows the femoral head associated with the pelvis to form a pathological joint (picture adapted from American Academy of Orthopaedic Surgeons web page, see Appendix IV). B: Picture shows the unsmooth appearance and uneven distribution of cartilage coating the femoral head from an OA pathological joint.

The damage to cartilage structure in arthritic disease is due to the activity of metabolites and inflammatory mediators that change the structural integrity of the cartilage matrix (Jeffrey, 1994). To explore this suggestion further, it is necessary to review the ultra-structure of cartilage and interactions with metabolites and inflammatory mediators that may promote the catabolic process.

1.1.3: Cartilage

Cartilage is a form of avascular connective tissue that contains chondrocytes within a specialised extracellular matrix. Cartilage is composed of 90-95% type II collagen with small amounts of types V, VI, IX, X and XI collagen also present in the cartilage matrix (Silver and Glasgold, 1995). Collagen fibrils form a cartilaginous framework that provides tensile strength. The collagen matrix consists of water (60-80% wet weight), collagen (10-20% wet weight), proteoglycans (10-15% wet weight) and other components such as adhesive molecules and lipids (Studer *et al.*, 1996). Load bearing capacity of articular cartilage is made possible through regional changes in water concentration which allow deformation of the cartilage surface in response to mechanical stress (Jeffrey, 1994) . Proteoglycans facilitate hydration of cartilage by producing an osmotic potential that maintains the water content (Bottomley *et al.*, 1997).

1.1.4: Proteoglycans in cartilage

The most abundant proteoglycan in articular cartilage is named aggrecan (Billington *et al.*, 1998) representing up to 10% of the dry weight. Aggrecan is a glycoprotein containing aggregated glycosaminoglycan (GAG) chains (Bolton *et al.*, 1999). Many individual monomers of aggrecan bind to hyaluronic acid to form an aggregate. The aggregate monomer is termed aggrecan (see Fig 1.1.4).

Fig. 1.1.4: Aggrecan structure

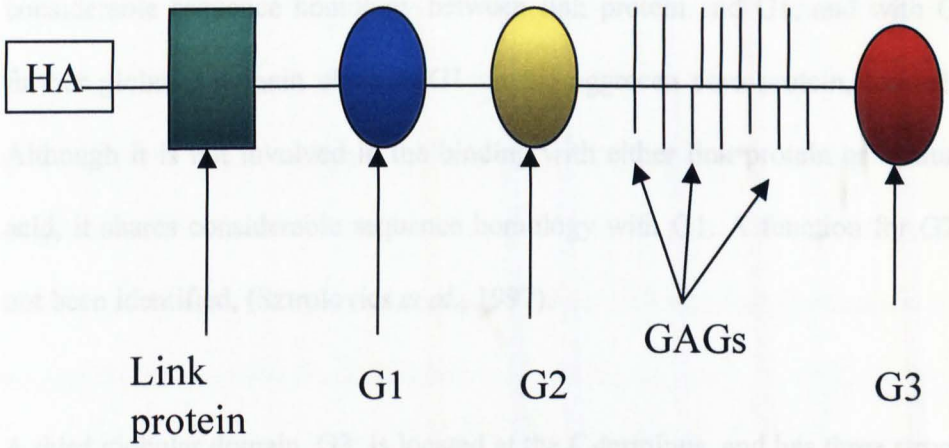


Fig. 1.1.4: Diagram shows structure of aggrecan. Aggrecan is bound non-covalently to hyaluronan (HA) and a stabilised link protein. Aggrecan is composed of G1, G2 individual sulphated GAGs and a G3 domain associated with a protein core. The sulphated GAGs produce an osmotic potential that maintains hydration of the cartilage matrix and therefore increases its ability to withstand compressive forces (diagram produced using Microsoft Powerpoint 97).

These aggregates are comprised of up to 100 monomers attached to a single chain of hyaluronic acid (HA). An aggrecan monomer consists of a protein backbone of 210-250 kDa to which is attached both chondroitin sulphate and keratan sulphate chains (Hering *et al.* 1997). The chains are attached to the central portion of the core protein, chondroitin sulphate chains (100-150 per monomer), being located in the C terminus and keratan sulphate is preferentially located towards the N terminus (Caterson *et. al.*, 2000). Individual aggrecan monomers, up to 100,

interact with hyaluronic acid to form a high molecular weight aggregate. This interaction involves a globular domain at the N-terminus, termed G1 or the hyaluronic acid binding region (HABR). The interaction is stabilised by a short protein called link protein which interacts with both the HA and G1. There is considerable sequence homology between link protein and G1, and with G2, a further globular domain close to G1 on the aggrecan core protein, termed G2. Although it is not involved in the binding with either link protein or hyaluronic acid, it shares considerable sequence homology with G1. A function for G2 has not been identified, (Sztrolovics *et al.*, 1997).

A third globular domain, G3, is located at the C-terminus, and has three structural domains: an EGF repeat, a lectin-like sequence, and a region homologous to the complement regulatory protein (CRP) motif. The lectin-like domain appears to be present in all forms of the molecule, while the EGF and CRP like domains are only present in an alternatively spliced variant. The roles of the various components of G3 have not been determined (Iozzo, 1998).

While aggrecan is found in cartilage, there are considerable similarities emerging between it and several other large proteoglycans. There are homologies with versican and the hyaluronate receptor CD44. Versican has a C-terminal lectin like domain and EGF-like repeats along with a central GAG binding domain, which unlike aggrecan is glycosylated exclusively with chondroitin sulphate (Iozzo, 1998).

The primary role of aggrecan is to induce osmotic swelling and maintain hydration in the cartilage extracellular matrix. In this way aggrecan plays a crucial role in the normal function of articular cartilage. The extracellular matrix of articular cartilage is comprised of fibril forming collagens, aggrecan and many other important molecules. The presence of very large numbers of chondroitin sulphate chains within the aggrecan molecule generates an osmotic swelling pressure. It is this hydration that is essential to the load bearing function of articular cartilage. Aggrecan catabolism involves release of the chondroitin sulphate into synovial fluid, a process that may be upregulated during inflammatory processes in and around the cartilage matrix (Billington *et al.*, 1998).

Subunits of glycosaminoglycans (GAGs) compose the structure of the aggrecan molecule. GAGs within cartilage include chondroitin-4-sulfate, chondroitin-6-sulfate and keratan sulfate. These molecules are bound to a protein core by sugar bonds to form an aggrecan molecule. Aggrecan molecules are further stabilised by link proteins which bind them to hyaluronan to form a proteoglycan aggregate.

Loss of proteoglycans from the cartilage matrix cause reduced hydration and therefore reduce the load bearing capacity. Chondrocytes embedded in the cartilage matrix are responsible for the turnover of these proteoglycan moieties. Chondrocytes both synthesise and facilitate the removal of proteoglycans in the cartilage matrix. Articular cartilage has been shown to be a heterogenous tissue that has varying composition with respect to both proteoglycans and chondrocytes

when moving from through the articular surface toward the epiphyseal growth plate (Bayliss *et al.*, 1983).

1.1.5: Zonal variations in cartilage

Articular cartilage varies in ultrastructure and composition with distance from the articular surface (Siczkowski and Watt, 1990). It has been demonstrated that amounts of both chondroitin sulphate and keratan sulphate vary in different regions of human articular cartilage (Bayliss *et al.*, 1983). This variation in cartilage structure can be divided into four zones: superficial, intermediate, deep and calcified as described by Silver and Glasgold, (1995).

1.1.5.1: Superficial Zone

The upper zone of cartilage is the uppermost surface of extracellular matrix that is exposed to the synovial joint space. It is composed of abundant tangentially oriented collagen fibres with associated proteoglycans and concordant hydration. Immunostaining has shown that proteoglycans and link protein are present, however, cationic stains were more intense in deeper regions of cartilage (Poole *et al.*, 1989) Chondrocytes maintain a discoid flattened morphology, reside parallel to the surface and have low metabolic activity. Chondrocytes derived from the upper zone were shown to attach and spread more slowly *in vitro* than chondrocytes deeper within the cartilage structure (Siczkowski and Watt, 1990).

1.1.5.2: Intermediate Zone

The intermediate zone is the layer below the superficial sliding zone. This is constructed of thicker collagen fibrils with chondrocytes arranged singly or in pairs. Cationic staining suggests that proteoglycan levels are elevated in this region compared to the upper zone (Poole *et al.*, 1987).

1.1.5.3: Deep Zone

The deep zone contains larger collagen fibrils with vertical (radial) orientation compared to the other zones. Chondrocytes in this region are aligned perpendicular to the joint surface, (Poole *et al.* 1984).

1.1.5.4:Calcified Zone

The calcified zone is defined by a transition between unmineralised and calcified cartilage. Matrix vesicles appear to bud off from the ends of chondrocyte cytoplasmic processes and may play a role in the mineralisation process in this region, (Silver and Glasgold, 1995). Activity of chondrocytes in cartilage matrix may be the key factor in regulation of cartilage functional capacity (Siczkowski and Watt, 1990).

1.1.6: Chondrocytes

Chondrocytes in cartilage are essential to the functional stability of the matrix. Chondrogenesis, the formation of articular cartilage and growth, is initiated by chondroprogenitor cells that aggregate and synthesise ECM (Siczkowski and Watt, 1990). These cells eventually become separated and surrounded by extracellular matrix. During the initial stages of development the cell density is high, and large volumes of ECM are synthesized. As the articular cartilage matrix structure is formed the cells proliferation and ECM synthesis is maintained to sustain a continual turnover of matrix without a change in volume (Buschmann *et al.,* 1992). At this stage chondroprogenitor cells have differentiated into chondrocytes (Mankin, 1974) (Fig. 1.1.6).

Fig. 1.1.6: Chondrocytes in the cartilage matrix

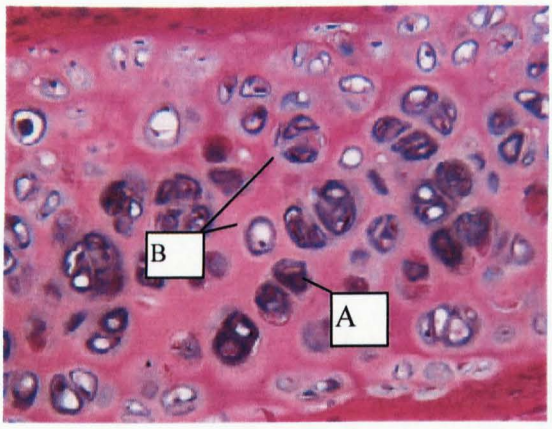


Fig. 1.1.6: Image of H&E stained chondrocytes within the cartilage matrix. A: Chondrocyte in the cartilage matrix. Chondrocytes are surrounded by a halo of light pink staining proteoglycans. Picture adapted from Microanatomy Web Atlas, (Appendix IV).

The matrix is a dynamic structure that is constantly subjected to both mechanical abrasion due to physical forces and chemical breakdown by release of endogenous proteases. Chondrocytes synthesize collagen and proteoglycans to maintain the integrity of the extracellular matrix. It has been suggested that mechanical forces on cartilage may play an important role in the anabolic activity of chondrocytes in the matrix (Hammrick, 1999). Chondrocytes have also been shown to induce the removal of proteoglycans and collagens from the matrix. This observation has been noted when articular cartilage is treated with inflammatory mediators such as IL-1β (Sandy *et al.*, 1995) and TNF-α (Homandberg *et al.*, 1996). Chondrocytes also express CD44 as a primary receptor for hyaluronan. Hyaluronan is responsible for the retention and orientation of proteoglycans in the cartilage matrix (Aguiar *et al.*, 1999). Levels of hyaluronan in the matrix and its

interactions with proteoglycans may be directly regulated by chondrocytes. Indeed, a study by Dsouza *et al.* (2000) revealed that both aggrecan and hyaluronan metabolism in chondrocytes are differentially regulated by IL-1 and that hyaluronan concentrations in the ECM may regulate the stability of proteoglycan aggregates.

Studies have shown that chondrocyte membranes have an aggrecanase activity (Billington *et al.*, 1998) that catalyses the breakdown of aggrecan molecules and that chondrocytes produce soluble proteases termed metalloproteinases (MMPs) that facilitate collagen (Wertheimer *et al.*, 1995) and proteoglycan (Bottomley *et al.*, 1997) loss from cartilage. Chondrocytes also produce other mediators that may facilitate GAG loss from articular cartilage such as nitric oxide (Evans and Stefanovicic, 1996) and prostaglandin-E_2 (Prete *et al.*, 1997). It has also been suggested that NO facilitates increased MMP activity in cartilage (Murrell *et al.*, 1995).

1.1.7: Nitric Oxide and cartilage function

Nitric oxide is a short lived, gaseous free radical, synthesized in the process of enzymic deamination of L-arginine to L-citrulline by NO synthases (NOS) (Nathan, 1994). There are three isoforms of NOS. Two forms are constitutive forms, eNOS and nNOS, the third type is an inducible isoform named iNOS. (Stuehr, 1997). NOS enzymes are dimeric in structure, calmodulin independent (iNOS) or possess binding domains (eNOS and nNOS), bear both FAD and FMN

and require tetrahydrobiopterin as a cofactor to produce NO (Nathan and Xie, 1994).

The *in vivo* synthesis of NO and its role in animal models of arthritis and human inflammatory joint disease may facilitate the development and maintenance of inflammatory conditions (Stichtenoth and Frolich, 1998). Articular chondrocytes have been shown to produce NO in response to cytokines and lipopolysaccharide (Stadler *et al.*, 1991).

In a study by Stichtenoth *et al*, (1994) its was demonstrated that adjuvant induced arthritis in rats caused an increase in urinary nitrate output compared to normal controls. Treatment of rats with L-nitro-arginine-methyl-ester (L-NAME), a non-specific inhibitor of iNOS reduced urinary nitrate excretion in adjuvant arthritic rats and reduced disease severity. A study by Farrell *et al.* (1992) showed that in human patients suffering from rheumatoid and osteoarthritis, serum and synovial fluid concentrations of nitrite were elevated compared to controls. Further evidence for involvement of NO in inflammatory diseases was produced by Kaur and Halliwell (1994) who showed that nitrotyrosine concentrations were elevated in serum and synovial fluid of patients with RA. Nitrotyrosine may be formed as a reaction product of peroxynitrite with tyrosine residues. Peroxynitrite is a shortlived highly reactive intermediate generated by NO degradation to nitrite (Vand der Vliet *et al.*, 1994).

There is evidence that links lipid peroxidation by reactive oxidative species (ROS) to cartilage matrix protein degeneration (Tiku *et al.*, 2000). In this study, ROS were generated and combined with anti-oxidants in the presence of chondrocytes and matrix proteins. It was found that the release of chondrocyte matrix components mediated by ROS was suppressed by the presence of vitamin E. The authors quantified peroxidation in this study by measuring *cis*-parinaric acid and concluded that ROS may regulate the pathogenesis of cartilage degeneration.

There is evidence that nitric oxide may have a direct role in facilitating the release of proteoglycans from the cartilage matrix. A study by Hassan *et al.*, (1998) showed that an artificial donor of nitric oxide, S-nitroso-N-acetyl-penicillamine (SNAP), caused increased degradation of GAGs *in vitro*. The report suggested that nitric oxide may facilitate degradation of GAGs directly and, or, peroxynitrite may also contribute to GAG degradation.

NO may also activate metalloproteinase (MMP) enzymes in articular cartilage (Murrell *et al.* 1995). The conclusions of this study suggest that MMP activity was reduced using L-NAME. The activity of MMPs was restored by addition of exogenous NO.

Many studies of NO in inflammatory models also consider the inter-relationship between NO and PGE_2 production and activity. These include studies of human chondrocytes (Blanco and Lotz, 1995), human osteoarthritic cartilage (Amin *et al.*, 1997) and rheumatoid synovial cells (Honda *et al.*, 2000).

1.1.8: Prostaglandin-E_2 and cartilage function

Prostaglandins are produced from polyunsaturated fatty acids by a series of reactions involving oxygenation, cyclization and the generation of five chiral centers from an achiral substrate (Marnett *et al.*, 1999). The first two stages in the prostaglandin biosynthetic pathway are catalysed by the enzymes cyclooxygenase (COX) 1 and 2 from the substrate arachidonic acid (AA). AA is transformed into primary prostanoids by differential enzymes and PGE isomerase is responsible for the generation of PGE_2 (Vane *et al.*, 1998).

COX 1 is a constitutive isoform, whereas COX 2 is an inducible isoform (Vane *et al.*, 1998). Both enzymes have a molecular weight of 71 KDa and possess 63 % sequence homology (Vane *et al.*, 1998). Willoughby *et al.*, (2000) have speculated the existence of a COX-3 enzyme, however, this has not been substantiated. Many NSAIDs used to reduce symptoms of RA have been shown to inhibit the activity of COX enzymes (Emery, 1999). PGE_2 is a product of these enzymes that has been studied in several models relating to inflammatory joint disease.

A study by Blanco and Lotz (1995) revealed that chondrocytes producing PGE_2 in response to treatment with IL-1β showed an increased level of cell proliferation. This effect was reduced by addition of indomethacin, a non-specific inhibitor of COX activity. This study also showed that IL-1β induced NO production, which

in turn, induced PGE_2 production in the chondrocytes. PGE_2 production induced by IL-1β was inhibited by addition of N^G-monomethyl arginine (L-NMMA), a NOS inhibitor. Thus, a link was established between PGE_2 and NO production with regard to chondrocyte monolayers *in vitro.*

The association between NO and PGE_2 was reviewed by Sautebin *et al.*, (1998). Indeed, similarities have been demonstrated in the NOS and COX pathways. Both enzymes have constitutive and inducible isoforms, are inhibited by glucocorticoids and induced by LPS and inflammatory cytokines.

Amin *et al.* (1997) showed that there was an apparent link between PGE_2 and NO in osteoarthritic cartilage. This study suggested that inhibition of NO by L-NMA, caused a reduced levels of PGE_2 in tissue culture supernatants. The authors concluded that NO may inhibit the production of endogenous PGE_2 in osteoarthritic cartilage explants. The studies by Blanco and Lotz (1995) and Amin *et al.* (1997) revealed relationships between PGE_2 and NO in chondrocytes and cartilage explants, respectively. The activity of COX-2 in chondrocytes via IL-1β induction has been linked to an intracellular signalling pathway involving C/CAAT-enhancer binding protein (Thomas *et al.*, 2000).

Induction of COX expression was also examined in rheumatoid synovial cells (Honda *et al.*, 2000). It was suggested that SNAP, an exogenous donor of NO, could induce expression of COX-2 in these cells. It was concluded by the authors

that increased COX-2 expression may exacerbate inflammatory responses through production of mediators such as prostaglandins.

The effect of prostaglandin-E_2 on cartilage *in vivo* is uncertain. Indeed, it may have a catabolic effect by increasing expression of other inflammatory mediators in chondrocytes and cells surrounding the inflamed joint and increasing angiogenic potential. Conversely, it may have beneficial effects by increasing aggrecan synthesis (Armin *et al.*, 1997). However, it is apparent that IL-1β and TNF-α are pivotal in the induction of both PGE_2 (Blanco and Lotz, 1995), (Berenbaum *et al.*, 1996) (Armin *et al.* 1997) and NO (Stefanovic-Racic *et al.* 1997), (Armin *et al.* 1997), (Bird *et. al.* 1997) , (Badger *et al.* 1998) in articular cartilage.

1.1.9: Cytokines and cartilage function in *in vitro* models

Analysis of cytokine mRNA and protein in RA tissues has revealed that many cytokines such as IL-1β, TNFα, IL-6, GM-CSF are detected in elevated concentrations. Cytokines are local protein mediators involved in processes such as cell growth and activation, inflammation, immunity and differentiation. The destruction of cartilage observed in arthritic diseases is considered to be a consequence of production of MMPs in response to cytokines such as IL-1β and TNFα (Feldmann *et al.*, 1996). It has been demonstrated that inhibition of TNFα using anti-TNFα antibodies and inhibition of IL-1β production reduces joint

damage in murine *in vivo* models of arthritic disease. The effects of these cytokines were reviewed within *in vitro* models using cartilage chondrocytes and explants (sections 1.1.9.1, 1.1.9.2 and 1.1.9.3).

1.1.9.1: Effects of Interleukin-1 (IL-1) on chondrocytes and cartilage explants

IL-1 is a multi-functional cytokine that affects nearly every cell type and often in concert with other cytokines and mediators (Dinarello, 1996). IL-1 is produced in two 17kD variants, IL-1α and IL-1β, and is commonly associated with the inflammatory response. It has been shown that IL-1β may increase production of inflammatory mediators such as NO, PGE_2 and MMPs that may regulate cartilage breakdown.

IL-1β has been used in many studies to determine its effects on cartilage in *vitro*. Previous studies have demonstrated that cartilage derived from different species and locations react differently to cytokines within an *in vitro* environment. Previous studies using bovine cartilage explants (Spirito *et al.*,1995; Wertheimer *et al.*,1995; Hanglow *et al.*, 1995; Bonassar *et al.*,1997; Steinmeyer *et al.*, 1997; Badger *et al.*, 1998) and chondrocytes (Badger *et al.*, 1998; Dsouza *et al.*, 2000) have shown that IL-1β has multiple biological actions. Spirito *et al.*, (1995) showed that IL-1α caused 90 % release of proteoglycans from bovine nasal cartilage explants during the intial 7 days of culture followed by loss of collagen

by day 21 of culture. The effect of IL-1β in this study was inhibited by MMP inhibitors CGS 20723 and Ro 31-9790. Wertheimer *et al.*, (1995) demonstrated that the MMP, stromelysin, was expressed in bovine occipital cartilage following treatment with IL-1β. Bonassar *at al.*, (1997) demonstrated that IL-1β produced >90% GAG loss in bovine cartilage isolated from the femoropatellar groove; this effect was inhibited by tissue inhibitor of metalloproteinase (TIMP) and the MMP inhibitor (L-758,354). It was also shown by Bonassar *et al.*, (1997) that IL-1β caused an increased loss of hyaluronan from bovine explants. Steinmeyer *et al.*, (1997) also showed that IL-1α induced proteoglycan release from bovine metacarpophalangeal cartilage explants. This effect was inhibited by the synthetic MMP inhibitor Ro 31-4724. Badger *et al.*, (1998) showed that IL-1α increased the production of NO, expression of iNOS and p38 mitogen activated kinase (MAP kinase) in bovine cartilage explants derived from the carpel metacarpel joints and bovine chondrocytes. Inhibition of IL-1α induced p38 MAP kinase production, iNOS mRNA production and nitrite production was produced using SB 203580, a specific inhibitor of p38 MAP kinase. Hanglow *et al.*, (1995) showed that IL-1β increased proteoglycan loss from bovine occipital cartilage explants and this was enhanced by L-NMMA. D'Souza *et al.*, (2000) also showed that IL-1α caused reduced proteoglycan incorporation into matrix formed by bovine metacarpophalangeal chondrocytes. These studies demonstrated that IL-1 has many effects catabolic effects on bovine cartilage explants.

Studies have also been performed using lapine cartilage explants (Stefanovic-Racic *et al.*, 1997) and chondrocytes (Berenbaum *et al.*, 1996; Jikko *et al.*, 1998) using IL-1. Stefanovic-Racic *et al.*, (1997) demonstrated that lapine cartilage explants from knee and shoulder joints produced increased levels of NO and suppression of proteoglycan synthesis into the explants when treated with IL-1β. Inhibition of NO production induced by IL-1β using L-NAME inhibited the effect of NO on suppression of proteoglycan synthesis, but caused increased loss of proteoglycans into the culture media. Jikko *et al.*, (1998) produced evidence that supported the previous study, showing that IL-1β reduced sulphate incorporation into GAGs in lapine knee chondrocyte cultures grown on plates coated with type I collagen. Berenbaum *et al.*, (1996) demonstrated that lapine shoulder, knee and femoral chondrocytes also produced PGE_2 following treatment with IL-1β.

In contrast to the aforementioned study by Stefanovic-Racic *et al.*, (1997) using lapine cartilage explants, Bird *et al.*, (1997) produced contradictory data using equine cartilage metacarpophalengeal explants that showed inhibition of NO induced by IL-1β using L-NIO did not affect proteoglycan loss from cartilage explants.

Studies have also been performed using rat cartilage explants *in vitro* (Desa *et al.*, 1989; Seed *et al.*, 1993) showed that IL-1α did not induce release of GAGs into the culture media by rat femoral head cartilages. However, addition of rat neonatal skin fibroblasts to rat cartilage explants caused an increased loss of GAGs into the

culture media. Conversely, Seed *et al.*, (1993) showed that proteoglycans were released from rat femoral head cartilages following treatment with IL-1β and the MMP inhibitor, U27391, inhibited this effect. These studies using entire rat femoral head cartilages showed that rat cartilage may be resistant to IL-1β mediated catabolism *in vitro*.

Studies have showed that, like rat cartilage explants, human cartilage explants offer resistance to IL-1 mediated cartilage breakdown (Nietfield *et al.*, 1990; Seed *et al.*, 1993) showed that human femoral head cartilage explants derived from different patients varied in their ability to release GAGs into media following treatment with IL-1β. Nietfield *et al.*, (1990) demonstrated that human cartilage explants, of unstated origin in the body from autopsies, did not release increased concentrations of proteoglycans into the media following treatment with 20% IL-1α and 80% IL-1β. A study by Blanco and Lotz, (1995) did, however, show that human femoral head chondrocyte cultures produced NO in following treatment with IL-1β. This study also suggested that IL-1β increased chondrocyte proliferation via an NO and PGE_2 dependent pathway. These studies demonstrate that human cartilage explants may have increased variability in their responses to treatment with IL-1 due to variation of age of the patients from which they were obtained. Literature was also reviewed regarding the effects of TNFα and LPS on cartilage explants and chondrocytes (sections 1.1.9.2 and 1.1.9.3, respectively).

1.1.9.2: The effects of Tumour Necrosis Factor α (TNFα) on cartilage explants and chondrocytes

TNFα is a mediator of inflammation that activates leukocytes, enhances migration of inflammatory cells into the extracellular matrix and triggers production of inflammatory cytokines (Tracey and Cerami, 1994). It may also contribute to the degradation of cartilage in arthritis disease. The use of TNFα on cartilage explant models (Stadler *et al.*, 1991) and chondrocytes (Berenbaum *et al.*, 1996) has been limited compared to studies using IL-1β.

Stadler *et al.*, (1991) showed that TNFα did not increase NO production by lapine knee and shoulder cartilage. Berenbaum *et al.*, (1996) demonstrated that TNFα did not increase production of PGE_2 in chondrocyte cultures isolated from lapine knee and shoulder cartilage. However, it was shown the TNFα combined with IL-1β increased production of NO (Stadler *et al.*, 1991) and PGE_2 (Berenbaum *et al.*, 1996). These studies indicated that TNFα may increase the responsiveness of cartilage explants and chondrocytes to other cytokines, such as IL-1β, using *in vitro* models. Previous studies have also indicated that LPS contributes to production of inflammatory mediators and cartilage breakdown using *in vitro* models (section 1.1.9.3)

1.1.9.3: The effects of lipopolysaccharide (LPS) on cartilage explants and chondrocytes

Components of the bacterial cell wall linked to infection may cause breakdown of the cartilage matrix by stimulation of macrophages *in vivo* to produce IL-1β and TNFα (Dinarello, 1996). Studies *in vitro* have also indicated that LPS may directly interact with cartilage explants to cause production of inflammatory mediators (Stadler *et al.*, 1991) and loss of proteoglycans from the cartilage matrix (Morales *et al.*, 1984; Ikebe *et al.*, 1993). Stadler *et al.*, (1991) showed that LPS caused increased production of NO and PGE_2 by chondrocytes isolated from lapine knee and shoulder cartilage. Morales *et al.*, (1984) demonstrated that bovine metacarpophalangeal cartilage explants synthesised reduced levels of proteoglycans, increased PGE_2 production and produced an increase in loss of proteoglycans into the culture media following treatment with LPS. Ikebe *et al.*, (1993) showed that similar inhibition of proteoglycan synthesis occured in rat costal chondrocytes following treatment with LPS. The combined results of these studies showed that LPS may exert similar effects on cartilage explants and chondrocytes compared to IL-1.

It has been shown that in RA tissue *in vivo*, macrophages have been identified as a source of IL-1β, TNFα and CSFs. It has been postulated that CSFs may orchestrate the cellular interactions and resulting events leading to cartilage breakdown (Hamilton, 1993).

1.2.0: Colony Stimulating Factors (CSFs): a subfamily of cytokines involved in inflammation.

The colony stimulating factors (CSFs) are a family of polypeptide growth factors critical to the development of haematopoeitic cells (Metcalf, 1989). The soluble colony stimulating factors include M-CSF (macrophage-colony stimulating factor, 44KDa), G-CSF (granulocyte-colony stimulating factor, 18-22 KDa), GM-CSF (granulocyte macrophage-colony stimulating factor, 22KDa) and IL-3, (also called multi-CSF, 14-30 KDa). Colony stimulating factors may act as differentiation factors initiating the irreversible terminal differentiation of progenitor cells. The proliferation of mature cells is usually not influenced by these factors, they may, however, prolong their life span. Apart from their actions on a number of hematopoietic cell types, CSFs also influence other cell types including those found within inflammatory processes Silvennoinen and Ihle, (1996). Indeed, CSFs have been used therapeutically to reduce neutropennia in Felty Syndrome (Vose and Armitage, 1995).

1.2.1: A role of CSFs with other inflammatory cytokines in arthritic disease?

It has been demonstrated that the CSFs have the ability to increase the sensitivity of the immune system and interact directly with a plethora of cell types (Nemunaitis, 1997). The role of CSFs in inflammation processes has not been fully characterised, however, GM-CSF / G-CSF (Campbell *et al.*, 1991) and M-

CSF (Campbell *et al.*, 1993) are produced by human cartilage and chondrocytes in response to stimulation with IL-1β. IL-3 has not been demonstrated to be produced by cartilage to date, however, IL-3 may be produced by other cells types *in vivo* during inflammation. The main aim of this study is to determine if CSFs have a direct role in the regulation of cartilage catabolism and surrounding tissue that may exacerbate inflammation and tissue damage *in vivo.* Indeed, it has been demonstrated that GM-CSF may be upregulated by tumour necrosis factor α (Haworth *et al.,* 1991) and that the GM-CSF receptor is expressed in synovial tissue (Berenbaum *et al.*, 1994). Furthermore, it has also been demonstrated in a recent study that both M-CSF and GM-CSF exacerbate inflammation in a murine *in vivo* model of joint inflammation (Bischof *et al.*, 2000).

It is possible that inflammatory mediators such as cytokines, including CSFs, may induce cells to produce inflammatory responses via overlapping intracellular signal transduction mechanisms. Thus, a link may be found between the functionality of cytokines in a given system due to interactions within intracellular signalling pathways.

1.2.2: Cytokine Signal Transduction

It has been shown that IL-1β induces intracellular signaling via p38 MAP kinase (Badger *et al.*, 1998) and C/CAAT enhancer binding protein (C/EBP) (Thomas *et al.*, 2000) in chondrocytes and nuclear factor kappaB (NF-κB) in inflamed synovial tissue (Marok *et al.*, 1996). It has also been shown that there is cross talk

between transcriptional factors NF-kB and C/EBP in the transcriptional regulation of genes (Xia *et al.*, 1997). Other cytokines such as IL-6 signal through a signal transducer and activator of transcription (STAT) pathway and have been shown to have similar effects as interleukin-1β (Ochrietor *et al.*, 2000). Indeed, IL-6 has been implicated in a recent study as a mediator of proteoglycan metabolism in articular cartilage (Flannery *et al.*, 2000). Like IL-6, G-CSF utilises an intracellular signaling pathway via STAT3 (Leornard and O'Shea, 1998) and that G-CSF activates p38 MAP kinase and extracellular signal-related kinase (ERK) pathways in hemopoietic cells (Rausch and Marshall, 1999). This study also suggests that IL-3 may also activate these signaling intermediates in hemopoietic cells. It is therefore possible that CSFs, such as G-CSF and IL-3, may influence the role of cells in the inflamed synovial joint via the aforementioned pathways. Cytokine signaling pathways that may influence cartilage breakdown and inflammation are illustrated in Fig. 1.2.2.

Fig. 1.2.2: Diagram showing cytokines and associated intracellular signaling pathways

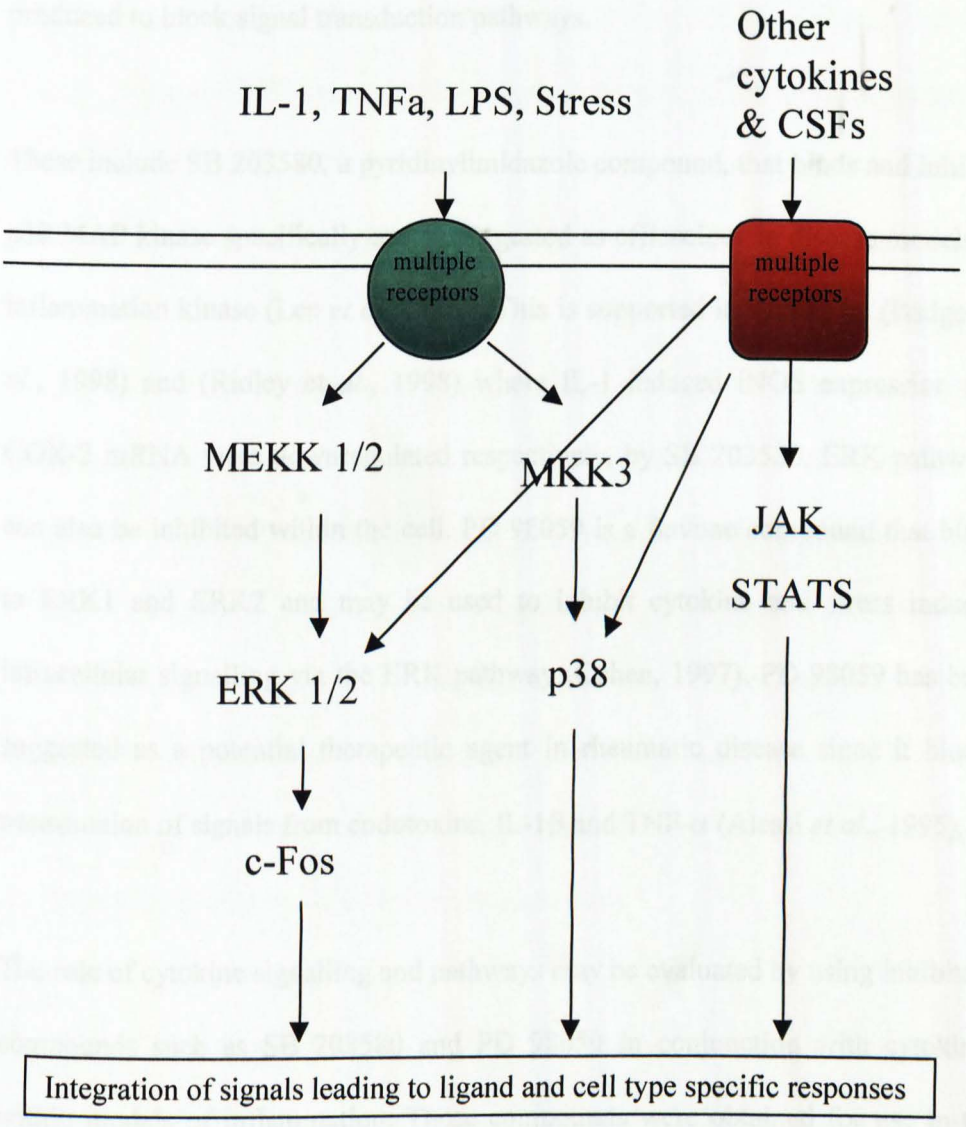


Fig 1.22: Diagram shows the cross talk between cytokines and the possibility of interactions between cytokines in producing a biological effect (diagram produced using Microsoft Powerpoint 97).

Cytokine signaling pathways have been identified as a possible therapeutic target for intervention in arthritic disease pathology to supress the inflammatory process. Indeed, both p38 MAP kinase (Lee *et al.*, 2000) and ERK 1/2 (Firestein and Manning, 1999) have been suggested as possible therapeutic targets. Indeed a number of specific inhibitors of signal transduction intermediates have been produced to block signal transduction pathways.

These include SB 203580, a pyridinylimidazole compound, that binds and inhibits p38 MAP kinase specifically and is suggested as efficacious in disease models of inflammation kinase (Lee *et al.*, 2000). This is supported in studies by (Badger et *al.*, 1998) and (Ridley et *al.*, 1998) where IL-1 induced iNOS expression and COX-2 mRNA were downregulated respectively, by SB 203580. ERK pathways can also be inhibited within the cell. PD 98059 is a flavone compound that binds to ERK1 and ERK2 and may be used to inhibit cytokine and stress induced intracellular signalling via the ERK pathway (Cohen, 1997). PD 98059 has been suggested as a potential therapeutic agent in rheumatic disease since it blocks transduction of signals from endotoxins, IL-1β and TNF-α (Alessi *et al.*, 1995).

The role of cytokine signalling and pathways may be evaluated by using inhibitory compounds such as SB 203580 and PD 98059 in conjunction with cytokines within models of inflammation. These compounds were obtained for use in the current study within a cartilage explant model system.

1.2.3: Objectives of this study.

The objective of this research was to produce a model of cartilage breakdown using cartilage explants. The primary aim was to assess different types of cartilage for release of GAGs into culture media and to select a cartilage type for studies of GAG release generation of nitric oxide and production of PGE_2 when treated with IL-1β, TNFα and LPS in an *in vitro* model (chapter 3). It was then planned to review the effects of CSFs on cartilage explants (chapter 4) and to study the combined effects of CSFs and IL-1β on cartilage explants (chapter 5).

A successive study was planned where cartilage explants were co-cultured with fibroblast monolayers *in vitro*. The aim of this study was to determine if fibroblasts would influence the effects of cytokines and CSFs on cartilage activity *in vitro* (chapter 7). Prior to commencing this study, fibroblast monolayers were analysed to measure production and release of GAGs, nitric oxide and PGE_2 modulated by cytokine and CSF treatments (chapter 6). The objective of this study was to provide data regarding fibroblasts interactions with cartilage explants and response to cytokines in the co-culture environment.

The final aim of this project was to study intracellular signaling pathways using specific inhibitors involved in cytokine signaling in both fibroblasts monolayers and cartilage explants and identify components that may regulate chondrocyte and fibroblast response to cytokines (chapter 8).

Chapter 3:

Establishing a cartilage explant model

3.1.0: Measurement of GAG release in articular and non articular cartilage explants

The objective of the preliminary investigation was to produce a profile of GAG loss in cartilage derived from different sources. In previous studies, models using both articular (Seed *et al.*, 1993; Hanglow *et al.*, 1995; Stefanovic-Racic *et al.*, 1997; Bird *et. al.*, 1997; Sandy *et al.*, 1999) and non articular cartilage (Spirito *et al.*, 1995; Bottomley *et al.*, 1997) have been used to measure *in vitro* GAG release in cartilage explant models. These studies have shown that pro-inflammatory mediators such as IL-1β and TNF-α induce GAG release from cartilage explants. Both load bearing and non-loading bearing cartilage samples were obtained to compare the extent of GAG loss when cultured as *in vitro* explants. This study was performed to determine if GAG loss varied in different cartilage types under control culture conditions.

Cartilage explants were dissected from human femoral heads, Male Wistar rats, porcine nasal and porcine podicep tissues (sections 2.1.3 & 2.1.4, respectively). Human femoral head cartilage explants were removed from tissue biopsies derived from fracture patients undergoing total hip replacement surgery (section 2.1.5). GAG concentrations were measured in explants immediately following dissection by digestion with papain (section 2.2.2) and in culture media as described in 2.3.3. The concentration of GAGs detected is illustrated in Table.

3.1.0 and compares the cartilage source, amount used, cartilage type and GAG concentration in explants before and after 6 days of *in vitro* culture. The change in GAG concentration was expressed as % GAG loss to allow comparison of GAG loss from cartilage explants of varying mass.

Table 3.1.0: Properties of cartilage explants from different sources.

	Cartilage Type			
Cartilage source	Wistar rat femoral head	Porcine metacarpo-phalangeal	Porcine Nasal	Human femoral head
Amount used	Femoral head (150-200mg)	Cartilage shavings (30-40mg)	Cartilage plugs (30-40mg)	Cartilage shavings (20-30mg)
Cartilage type	Articular	Articular	Non Articular	Articular
GAG concentration (pre-culture, μg/ml)	480 ± 40	476 ± 78	520 ± 58	356 ± 73
GAG concentration (post culture, μg/ml)	385 ± 48	270 ± 68	279 ± 69	260 ± 64
% GAG loss after 6 days of *in vitro* culture	20	43	46	27

Table: 3.1.0: Cartilage explants (n=10) were used to measure percentage GAG loss during 6 days in culture. Wistar rat, porcine metacarpo-phalangeal and human cartilage explants were classified as load bearing cartilage. Porcine nasal cartilage was classified as non-load bearing cartilage. Porcine nasal cartilage, porcine metacarpo-phalangeal cartilage, and human femoral head cartilage were as 30-40 mg explants from tissue biopsies. Pre-culture and post-culture GAG concentrations were expressed with ± SE of the mean.

Porcine nasal cartilage and porcine metacarpo-phalangeal cartilage showed a significant ($p<0.05$) loss of total GAG concentration during 6 days in culture. Porcine metacarpo-phalangeal cartilage and nasal cartilage explants lost 46 % and 43 % of GAGs respectively, during 6 days in culture. Human femoral head explants showed a loss of 27 % of total GAGs during 6 days in culture. Wistar rat cartilage showed a loss of 20% during 6 days in culture. GAG loss from both human and rat cartilage explants was less than that observed in porcine cartilage within control culture conditions. It was therefore concluded that rat or human cartilage explants would prove most useful for comparative studies on the effects of cytokines on GAGs release in this *in vitro* system. However, quantity and quality of cartilage explants available from human tissue samples proved to be highly variable during these preliminary studies, therefore rat femoral head cartilages were selected for use in further experiments.

Rat cartilage explants were selected as the explant type to use in experiments using inflammatory mediators to stimulate an increase of GAG release. Desa *et al*, (1989) showed that GAG content in Wistar rat cartilage explants was not affected by treatment with IL-1α. However, a subsequent study by Seed *et al.* (1993) showed that there was an increase in GAGs lost from rat femoral head cartilages in response to human recombinant IL-1β. IL-1β was therefore selected to test as a positive control in rat cartilage explant experiments. Rat FHC were also selected since they showed a low % GAG loss from cartilage explants after 6 days in control culture media conditions. It was considered that this would be useful when

differentiating GAG release mediated by inflammatory mediators from GAG release produced as a consequence of the *in vitro* explant culture method.

3.1.1 Optimisation of the tissue culture medium

Culture medium was initially used consisting of DMEM, HEPES buffer, 10% fetal calf serum (FCS), 2mM L-glutamine, 100μU/ml penicillin and 100μg/ml streptomycin. The constituents were collectively named complete supplemented media (CSM). DMEM was used without phenol red to allow measurement of GAGs released into the media using the DMB assay adapted from methodology described by Goldberg and Kolibas (1990). Absence of phenol red from the DMEM also allowed measurement of nitrite produced in the culture media using the Griess reaction adapted from methodology described by Verdon *et al.* (1995). FCS was added to maintain viability of chondrocytes within the cartilage explants and to supply essential nutrients and growth factors during 6 days of tissue culture. L-glutamine was added as an essential amino acid. Penicillin and streptomycin were added to reduce the potential of bacterial infection. It was noted that DMEM used in the explant culture contained sodium bicarbonate buffer. Therefore it was considered that HEPES buffer may not be essential to maintain pH in the CSM.

This was tested by culturing rat cartilages explants in the presence and absence of 2mM HEPES. The pH in the culture media was monitored to determine if 2mM HEPES buffer was a necessary additive to the CSM. The pH of the media following culture was compared in CSM containing 2mM HEPES and CSM

lacking HEPES (see Table. 3.1.1). The tissue culture media were measured to determine if the pH changed in CSM lacking HEPES compared to CSM containing 2mM HEPES.

Table. 3.1.1: pH measurements in CSM containing 2mM HEPES buffer compared to HEPES free CSM.

Media type	Day 3 pH	Day 6 pH
CSM + HEPES	7.2	7.1
CSM - HEPES	7.3	7.2

Table: 3.1.1: The presence of 2mM HEPES buffer in the CSM did not change the pH of CSM compared to CSM free HEPES.

HEPES did not cause a change or prevent a change in pH in the CSM during culture of rat cartilage explants for intervals of 3 days. HEPES was therefore omitted from the CSM for further experiments since DMEM pH was deemed to be sufficiently buffered by sodium bicarbonate within DMEM. Wistar FHCs were then cultured in optimised media to determine the effects of characterised proinflammatory mediators on GAG release, nitrite and PGE_2 production in the culture media.

3.1.2: Effect of IL-1β on GAG concentration in post culture cartilage explants

IL-1β is a soluble cytokine and proposed to be an important peptide in the regulation of cartilage destruction (Feldmann *et al.*, 1996; Bird *et al.*, 1997). Rat femoral head cartilage explants were treated with 1, 10 and 50 ng/ml of IL-1β to produce a dose response curve profile. GAGs were measured in the culture media to determine if rat femoral head cartilage showed increase loss of GAGs from release when treated with IL-1β. Nitrite production was measured in the culture media as a measure of NO production by treatment with IL-1β. PGE_2 concentrations were measured in the tissue culture media to determine if COX activity was regulated in rat cartilage explants by IL-1β. The concentration of GAGs in cartilage explants was measured after 6 days of tissue culture with 1, 10 and 50ng/ml IL-1β. The concentration of IL-1β used in subsequent experiments was further validated by determining an IC_{50} value by measuring production of nitrite by cartilage explants during 3 days (see Appendix V). The IC_{50} value calculated using this technique was ~10ng/ml. The concentrations 1-50 ng/ml were also selected to compare with equivalent concentrations of IL-1β used by Hanglow *et al.* (1995), Spirito *et al.* (1995), and Stefanovic-Racic *et al.* (1997). The concentrations of GAGs in cartilage explant digests following tissue culture were compared in Fig. 3.1.2.

Fig. 3.1.2: Effect of IL-1β on GAG content in rat femoral head cartilage

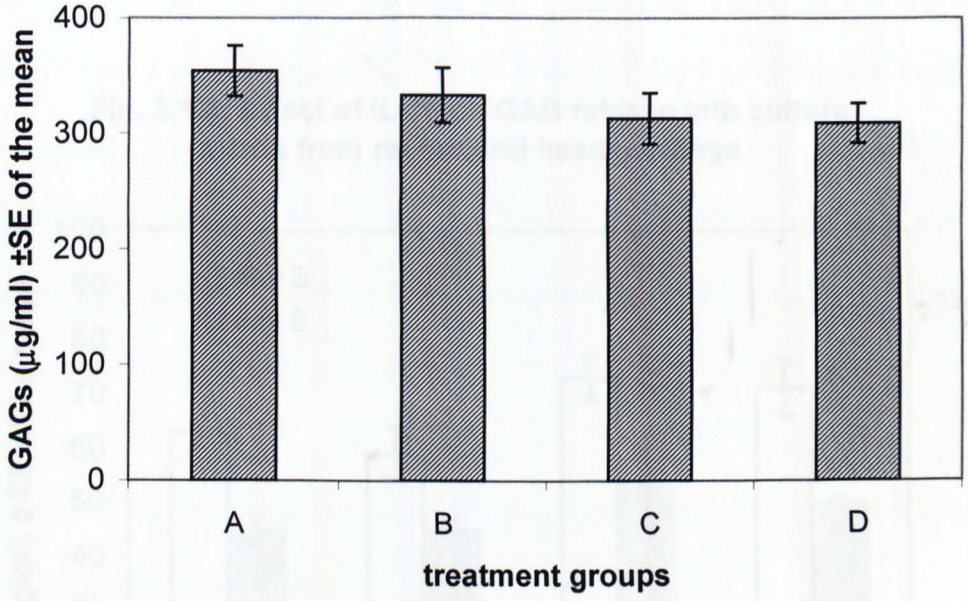


Fig. 3.1.2: Rat femoral head cartilage explants (n=8) were treated as follows: control (A), 1ng/ml IL-1β (B), 10ng/ml IL-1β (C) and 50ng/ml IL-1β (D) during a six day incubation. Culture media was changed on day 3 and cartilage GAGs were measured in explants following the 6 culture. Analysis of data using a Students t test showed that there was no significant difference was observed between control groups and treated groups.

GAG concentrations in cartilages did not change following treatment with IL-1β for 6 days (see Fig. 3.1.2). This experiment was repeated 3 times and produced the same result on each occasion. The GAG loss from cartilage explants was also measured in the culture media following treatment with 1, 10 and 50ng/ml of IL-1β. This was measured in culture media collected on days 3 and 6 (see Fig. 3.1.3).

3.1.3: Effect of IL-1β on GAG concentration released in the culture media by cartilage explants

Fig. 3.1.3: Effect of IL-1β on GAG release into culture media from rat femoral head cartilage

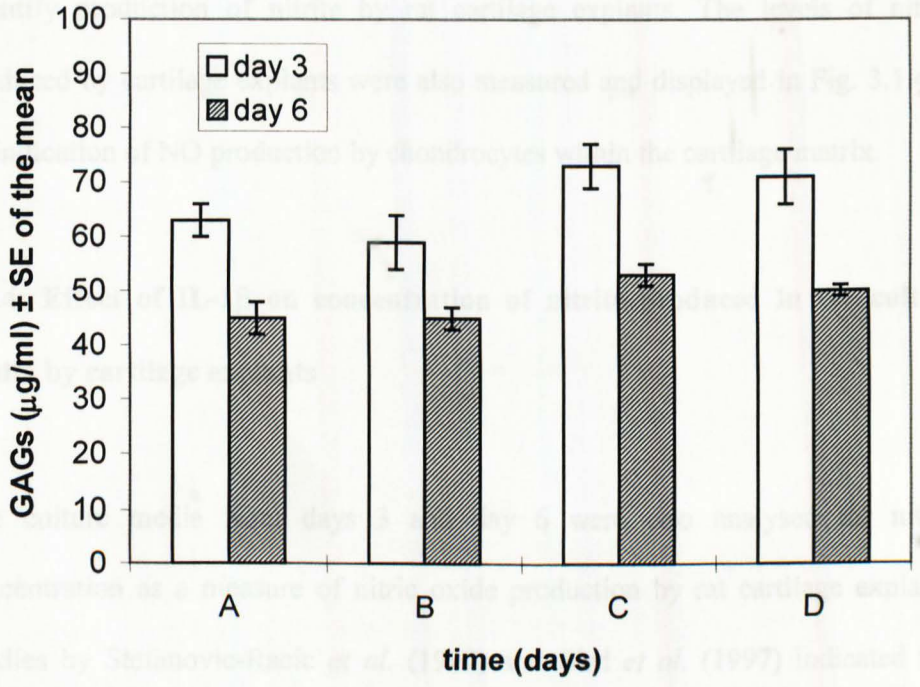


Fig. 3.1.3: Rat femoral head cartilage explants (n=8) were treated as follows: control (A), 1ng/ml IL-1β (B), 10ng/ml IL-1β (C) and 50ng/ml IL-1β (D) during a six day incubation. Culture media was changed on day 3. GAGs were measured in the culture media on days 3 and days 6. Analysis of data using a Students t test showed that there was no significant difference was observed between control groups and treated groups.

GAG concentrations released in the tissue culture media were not changed by treatment with 1, 10 and 50ng/ml of IL-1β during 6 days of culture. This was

surprising since research by Seed *et al.* (1993) showed that human recombinant IL-1β induced glycosaminoglycan loss from rat FHC. It was observed that GAG concentrations measured in the media at day 6 were reduced compared to GAG concentrations measured in media collected following day 3 in both control and IL-1β groups. Culture media collected from these experiments were also used to quantify production of nitrite by rat cartilage explants. The levels of nitrite produced by cartilage explants were also measured and displayed in Fig. 3.1.4 as an indication of NO production by chondrocytes within the cartilage matrix.

3.1.4: Effect of IL-1β on concentration of nitrite produced in the culture media by cartilage explants

The culture media from days 3 and day 6 were also analysed for nitrite concentration as a measure of nitric oxide production by rat cartilage explants. Studies by Stefanovic-Racic *et al.* (1997) and Bird *et al.* (1997) indicated that production of nitric oxide was increased by cartilage explants following treatment with IL-1β. Nitrite was measured in the media from rat FHC explant model to compare with these studies. Nitrite concentration was determined by the Griess reaction adapted from Verdon *et al* (1995) at days 3 and 6 in the tissue culture media, data obtained from this experiment are illustrated in Fig. 3.1.4.

Fig. 3.1.4: Effect of IL-1β on production of nitrite by rat femoral head cartilage

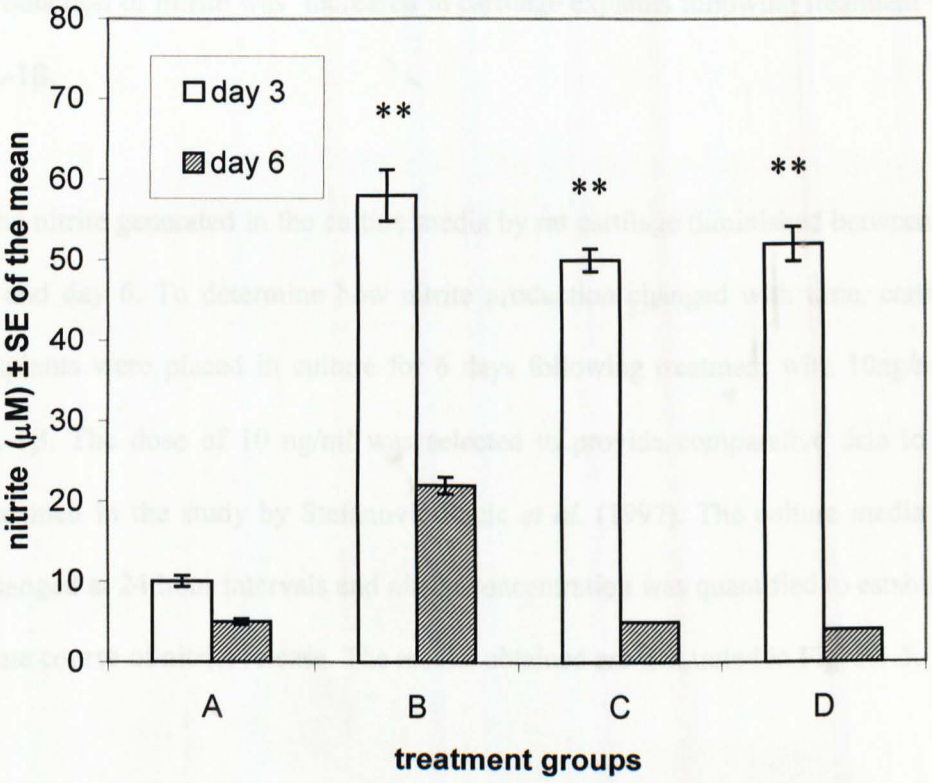


Fig. 3.1.4: Rat femoral head cartilage explants (n=8) were treated as follows: control (A), 1ng/ml IL-1β (B), 10ng/ml IL-1β (C) and 50ng/ml IL-1β (D) during a six day incubation. Culture media was changed on day 3. Nitrite concentration was measured in the culture media on days 3 and days 6. Analysis of data using a Students t test showed that IL-1β at 1, 10 and 50 ng/ml produced a significant (**$P<0.01$) increase in nitrite in the culture media after 3 days compared to respective same day controls.

Nitrite concentrations in culture media were significantly ($p<0.01$) increased by 1 ng/ml, 10 ng/ml and 50 ng/ml IL-1β compared to controls after 3 days in culture. However, nitrite concentrations in culture media at day 6 were not significantly

different from controls. These results were in accordance with studies by Stefanovic-Racic *et al.* (1997) and Bird *et. al.* (1997) which suggested that production of nitrite was increased in cartilage explants following treatment with IL-1β.

The nitrite generated in the culture media by rat cartilage diminished between day 3 and day 6. To determine how nitrite production changed with time, cartilage explants were placed in culture for 6 days following treatment with 10ng/ml of IL-1β. The dose of 10 ng/ml was selected to provide comparative data to that obtained in the study by Stefanovic-Racic *et al.* (1997). The culture media was changed at 24 hour intervals and nitrite concentration was quantified to establish a time course of nitrite release. The results obtained are illustrated in Fig. 3.1.5.

3.1.5: Effect of IL-1β on concentration of nitrite produced in the culture media by cartilage explants during a time course of 6 days

Fig. 3.1.5: Effect of IL-1β on nitrite production by rat femoral head cartilage during a 6 day time course.

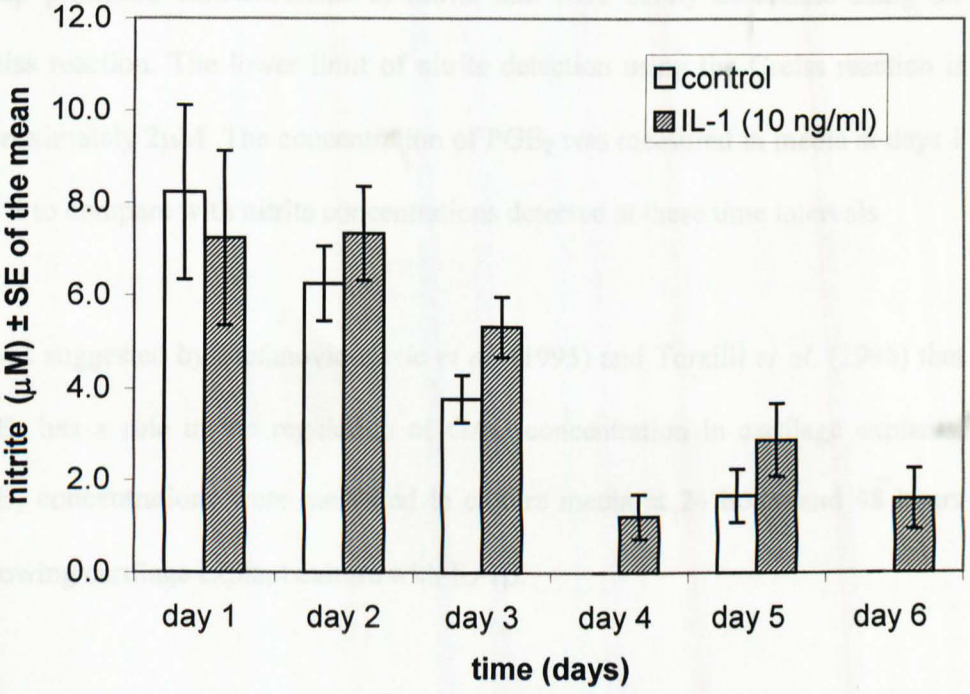


Fig. 3.1.5: A time course of nitrite production in tissue culture supernatant was measured during a 6 day period at 24 hour intervals following treatment of cartilage explants (n=6) with 10 ng/ml of IL-1β. Analysis of data using a Students t test showed that there was no significant difference between the IL-1β treated group and the control group at each time point. However, nitrite levels in both control and IL-1β groups were highest at day 1 and had decreased by day 3.

Nitrite concentrations in produced in the 10ng/ml IL-1β group showed no significant difference from same day controls in this experiment. The concentration of nitrite diminished in both control and 10 ng/ml IL-1β groups during the time course of six days. Nitrite levels were greatly reduced following three days in culture media. This trend was also illustrated in a study by Stefanovic-Racic *et al.* (1997). On days 4, 5 and 6 both the IL-1β and control group produced concentrations of nitrite that were barely detectable using the Greiss reaction. The lower limit of nitrite detection using the Greiss reaction is approximately 2μM. The concentration of PGE_2 was measured in media at days 1 and 2 to compare with nitrite concentrations detected at these time intervals

It was suggested by Stefanovic-Racic *et al.* (1995) and Torzilli *et al.* (1996) that PGE_2 has a role in the regulation of GAG concentration in cartilage explants. PGE_2 concentrations were measured in culture media at 24 hours and 48 hours following cartilage explant culture with IL-1β.

3.16: Effect of IL-1β on concentration of PGE_2 produced by cartilage explants

PGE_2 has been correlated with regulation of cartilage GAG concentrations in articular cartilage (Torzilli *et al.*, 1996) and chondrocytes (Blanco and Lotz, 1995; Berenbaum *et al.*, 1996). The concentration of PGE_2 was determined in the rat cartilage explant model by measuring levels in culture media following treatment

with 10 ng/ml of IL-1β. This concentration was selected to compare PGE_2 concentrations detected with quantified levels of nitrite as shown in Fig 3.1.5 after 24 and 48 hours. PGE_2 concentrations are illustrated in Fig. 3.1.6.

Fig. 3.1.6: Effect of IL-1β on PGE2 production in rat femoral head cartilage

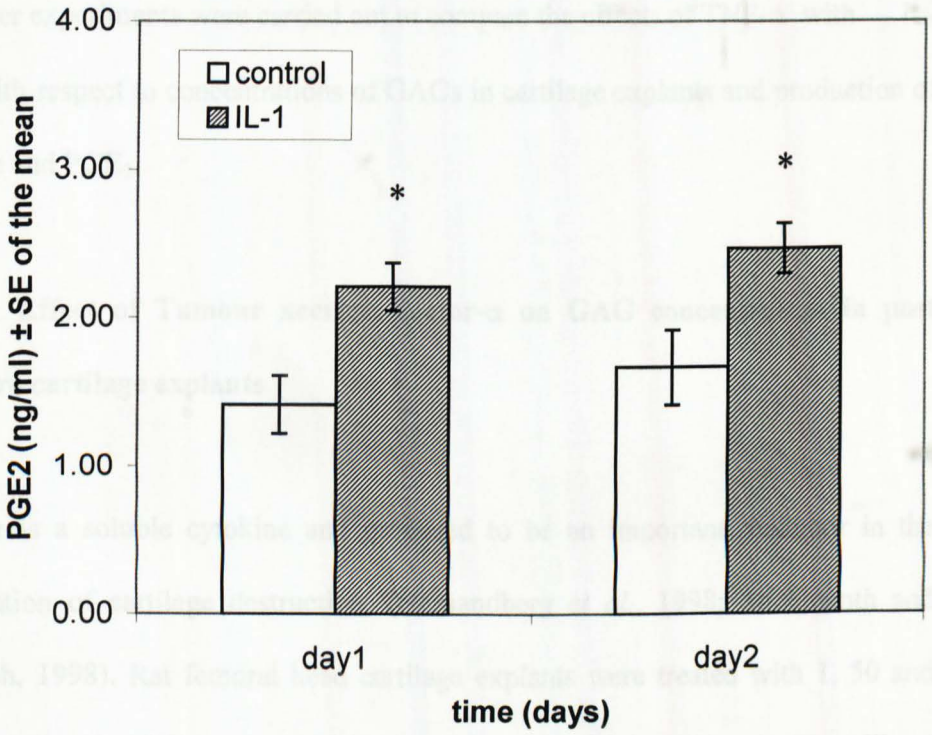


Fig. 3.1.6: Rat femoral head cartilage explants (n=8) were treated with 10ng/ml of IL-1β for a 48 hour incubation with a change of media at 24 hours. PGE_2 concentration was measured in the culture media and compared to controls. Analysis of data using a Students t test showed that PGE_2 concentrations were significantly ($P<0.05$) increased in the group treated with 10ng/ml of IL-1β at 24 and 48 hours compared to same day controls.

PGE_2 concentrations were significantly ($p<0.05$) increased in the group treated with IL-1β compared to the control group at both 24 and 48 hours. The data obtained from this study was consistent with studies by Blanco and Lotz (1995) and Berenbaum *et al.* (1996) which demonstrated that IL-1β caused increase production of PGE_2 in chondrocyte monolayer cultures.

Further experiments were carried out to compare the effects of TNF-α with IL-1β with respect to concentrations of GAGs in cartilage explants and production of nitrite and PGE_2.

3.1.7: Effect of Tumour necrosis factor-α on GAG concentration in post culture cartilage explants

TNFα is a soluble cytokine and proposed to be an important mediator in the regulation of cartilage destruction, (Homandberg *et al.*, 1998; Stichtenoth and Frolich, 1998). Rat femoral head cartilage explants were treated with 1, 50 and 100ng/ml of TNF-α to produce a dose response curve profile. These concentrations were selected to provide comparative data to compare with studies by Stadler *et al.* (1991) and Berenbaum *et al.*, (1996).

The role of TNF-α in the rat explant model was assessed by measuring GAGs in the culture media and digested cartilages following culture to determine if rat femoral head cartilage explants showed loss of GAGs when subjected to treatment

with TNFα. Nitrite production was measured in the culture media as an indicator of NO production mediated by TNFα. PGE_2 concentrations were also measured in the tissue culture media to determine TNFα produced similar effects to those observed with IL-1β, illustrated in Fig. 3.1.6.

The concentration of GAGs in cartilage explants was measured after day 6 of tissue culture with 1, 50 and 100ng/ml TNF-α. The concentrations of GAGs in cartilage explants digested following culture were compared in Fig. 3.1.7.

Fig. 3.1.7: Effect of TNF-α on GAG content in rat femoral head cartilage.

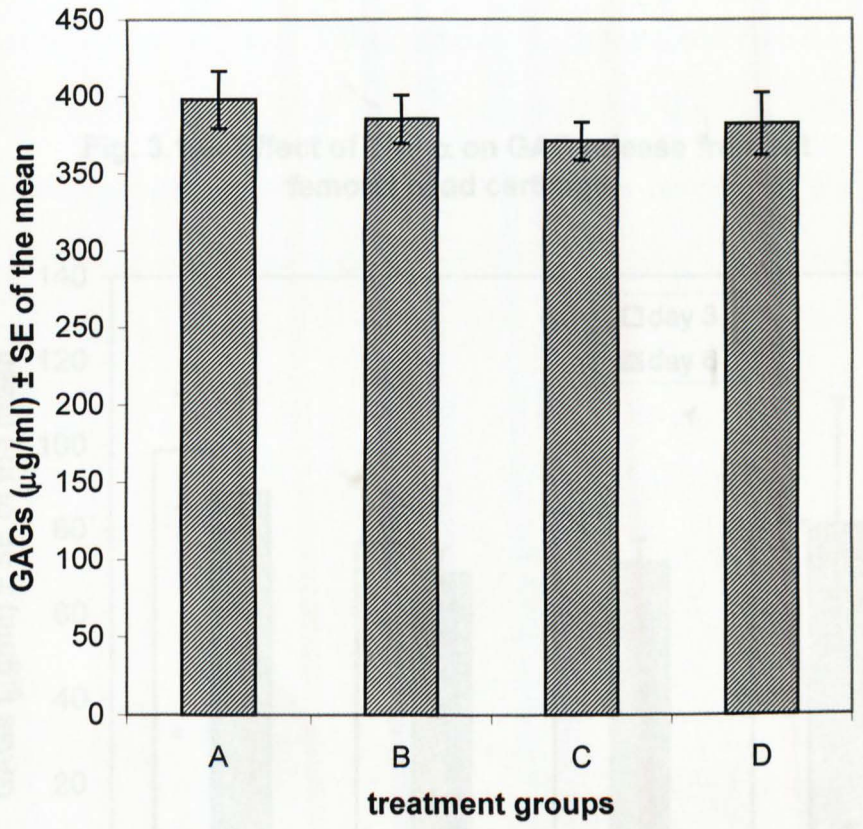


Fig. 3.1.7: Rat femoral head cartilage explants (n=8) were treated as follows: control (A), 1ng/ml TNFα (B), 50ng/ml TNFα (C) and 100ng/ml TNFα (D) during a six day incubation. Culture media were changed on day 3 and cartilage GAGs were measured in explants following culture. Analysis of data using a Students t test showed that there was no significant differences observed between control and treated groups.

GAG concentrations in cartilages did not change compared to controls following treatment with TNFα for 6 days (see Fig. 3.1.7). The GAG loss from cartilage explants was also measured in the culture media following treatment with 1, 50 and 100ng/ml of TNFα on days 3 and 6 (see Fig. 3.1.8).

3.1.8: Effect of TNFα on concentration of nitrite produced in the culture media by cartilage explants

Fig. 3.1.8: Effect of TNF-α on GAG release from rat femoral head cartilage

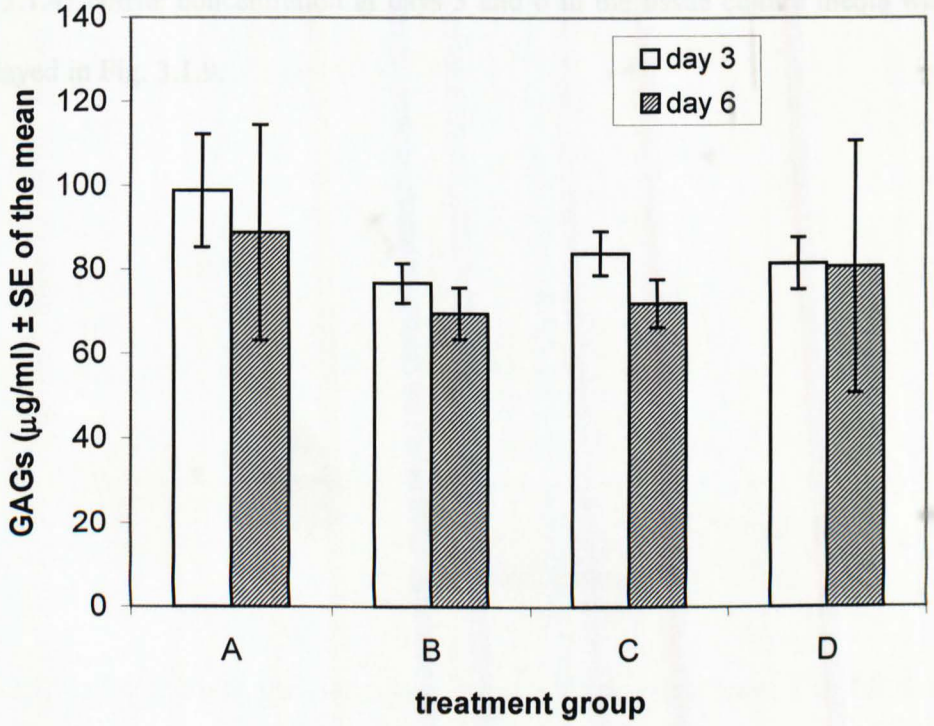


Fig. 3.1.8: Rat femoral head cartilage explants (n=8) were treated as follows: control (A), 1ng/ml TNFα (B), 50ng/ml TNFα (C) and 100ng/ml TNFα (D) during a six day incubation. Culture media was changed on day 3. GAGs were measured in the culture media on days 3 and days 6. Analysis of data using a Students t test showed that there was no significant differences were observed between control and treated groups.

GAG concentrations in the tissue culture media were not altered from controls by

treatment with 1, 50 and 100ng/ml of TNF-α during 6 days of culture. It was observed that GAG concentrations in the media at day 6 were lower than GAG concentrations observed at day 3 in all groups. The culture media from days 3 and day 6 were also analysed for nitrite concentration to compare with GAG concentrations and the results obtained using IL-1β in a comparative study (see Fig. 3.1.4) Nitrite concentration at days 3 and 6 in the tissue culture media was displayed in Fig. 3.1.9.

3.1.9: Effect of TNFα on concentration of nitrite produced in the culture media by cartilage explants

Fig. 3.1.9: Effect of TNF-α on nitrite production by rat femoral head cartilage.

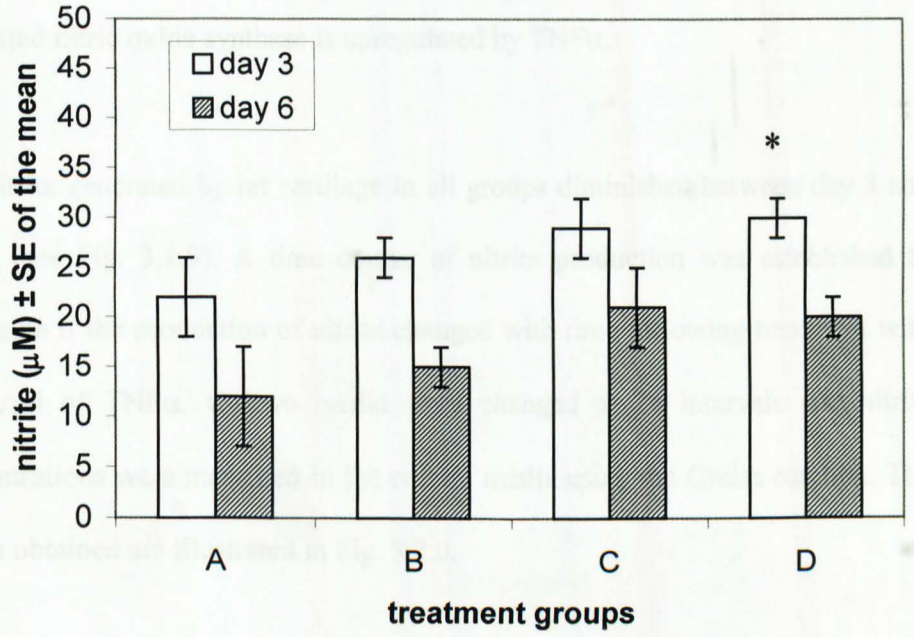


Fig. 3.1.9: Rat femoral head cartilage explants (n=8) were treated as follows: control (A), 1ng/ml TNFα (B), 50ng/ml TNFα (C) and 100ng/ml TNFα (D) during a six day incubation. Culture media were changed on day 3. Nitrite concentration was measured in the culture media on days 3 and days 6. TNFα concentration of 100ng/ml caused a significant ($P<0.05$) increase in nitrite concentrations in the culture media at day 3. Analysis of data using a Students t test showed that there was no significant differences observed between control groups and treated groups.

Nitrite concentrations in culture media were significantly ($p<0.05$) increased by 100ng/ml TNFα compared to controls after 3 days. Nitrite concentrations in culture media at day 6 were not significantly different from controls. This compared with studies by Stadler *et al.* (1991) that suggested chondrocytes do not release nitric oxide in response to TNFα and by Badger *et al.* (1998) that suggested nitric oxide synthase is upregulated by TNFα.

The nitrite generated by rat cartilage in all groups diminished between day 3 and day 6 (see Fig. 3.1.9). A time course of nitrite production was established to determine if the production of nitrite changed with time following treatment with 100ng/ml of TNFα. Culture media were changed at 24 intervals and nitrite concentrations were measured in the culture media using the Greiss reaction. The results obtained are illustrated in Fig. 3.2.0.

3.2.0: Effect of TNFα on concentration of nitrite produced in the culture media by cartilage explants during a time course of 6 days

Fig. 3.2.0: Effect of TNF-α on nitrite production by rat femoral head cartilage.

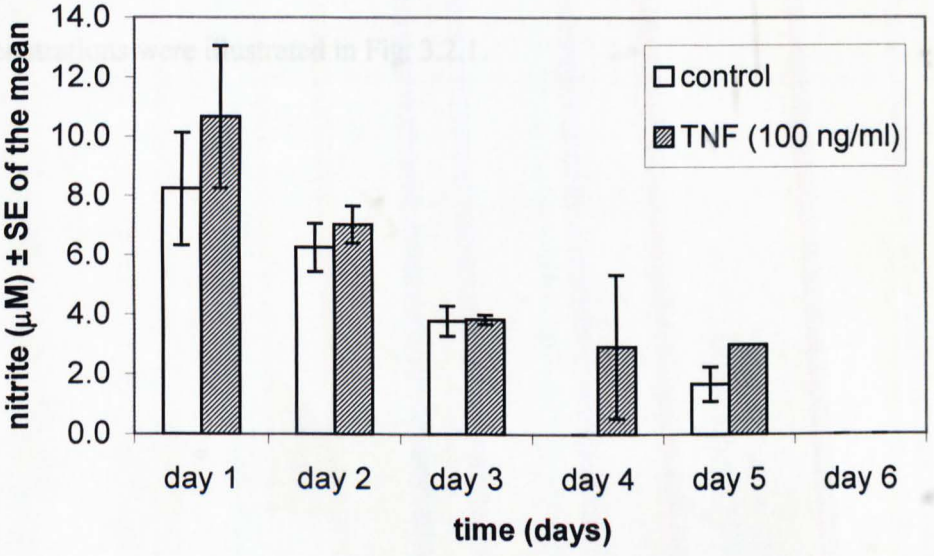


Fig. 3.2.0: A time course of nitrite production by rat femoral head cartilage (n=8) in tissue culture supernatant was measured during a six day period at 24 hour intervals following treatment with 100ng/ml of TNFα. In this experiment there was no significant difference between 100ng/ml TNFα and respective same day control groups. Analysis of data using a Students t test showed that there were no significant differences between control and treated groups.

Nitrite concentrations produced in the 100ng/ml TNFα group showed no significant difference from same day controls in this experiment (Fig. 3.2.0). The concentration of nitrite diminished in both control and 100 ng/ml TNFα groups

during the time course of six days. At days 4 and day 6 the control group did not produce detectable concentrations of nitrite using the Griess reaction.

PGE_2 concentrations were also measured in the culture media at days 1 and 2 following treatment with 100ng/ml TNFα. This study provided data that was comparable with nitrite concentrations at equivalent time points. PGE_2 concentrations were illustrated in Fig. 3.2.1.

3.2.1: Effect of TNF-α on concentration of PGE_2 produced by cartilage explants

Fig. 3.2.1: Effect of TNF-α on PGE2 production by rat femoral head cartilage.

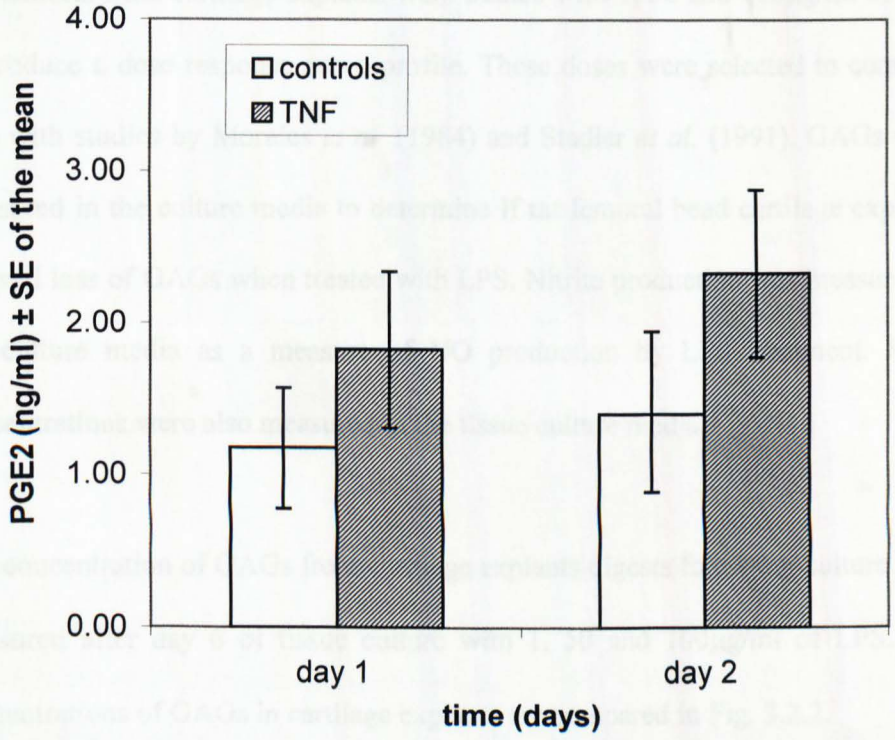


Fig. 3.2.1: Rat femoral head cartilage explants (n=8) were treated with 100ng/ml of tumour necrosis factor-α for a 48 hour incubation with a change of media at 24 hours. Analysis of data using a Students t test showed that there was no significant differences observed between control groups and treated groups.

PGE_2 concentrations were not significantly different in the 100 ng/ml TNFα group compared to the respective same day control groups. These results were consistent

with a study by Berenbaum *et al.* (1996). A study was also performed using LPS (endotoxin) as an inflammatory mediator to compare its effects on cartilage explants with those of IL-1β and TNFα.

3.22: Effect of LPS on GAG concentration in post culture cartilage explants

Rat femoral head cartilage explants were treated with 1, 50 and 100μg/ml of LPS to produce a dose response curve profile. These doses were selected to compare data with studies by Morales *et al.* (1984) and Stadler *et al.* (1991). GAGs were measured in the culture media to determine if rat femoral head cartilage explants showed loss of GAGs when treated with LPS. Nitrite production was measured in the culture media as a measure of NO production by LPS treatment. PGE_2 concentrations were also measured in the tissue culture media.

The concentration of GAGs from cartilage explants digests following culture were measured after day 6 of tissue culture with 1, 50 and 100μg/ml of LPS. The concentrations of GAGs in cartilage explants are compared in Fig. 3.2.2.

Fig. 3.2.2: Effect of LPS on GAG content in rat femoral head cartilage.

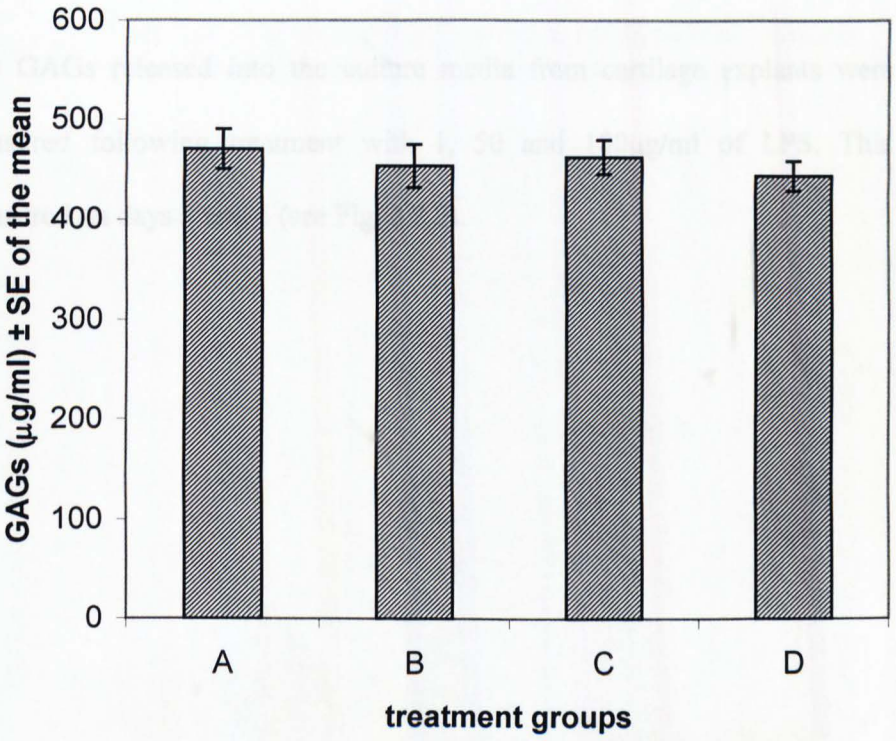


Fig. 3.2.2: Rat femoral head cartilage explants (n=8) were treated as follows: control (A) 1µg/ml LPS (B), 50µg/ml LPS (C) and 100µg/ml LPS (D) for a six day incubation. Culture media were changed on day 3. GAGs were measured in the cartilage explant digests following 6 days in culture. Analysis of data using a Students t test showed that there were no significant differences between control and treated groups.

There was no significant difference between GAG concentrations in cartilages following treatment with LPS for 6 days compared to controls (see Fig. 3.2.2). This experiment was repeated 3 times with similar results on each occasion. This was surprising since these results contradicted a study by Morales *et al.* (1984)

that showed that LPS induced a release of proteoglycans in cartilage explant cultures.

The GAGs released into the culture media from cartilage explants were also measured following treatment with 1, 50 and 100μg/ml of LPS. This was measured on days 3 and 6 (see Fig. 3.2.3).

3.2.3: Effect of LPS on GAG release into the culture media by cartilage explants

Fig. 3.2.3: Effect of LPS on GAG release into culture media from rat femoral head cartilage

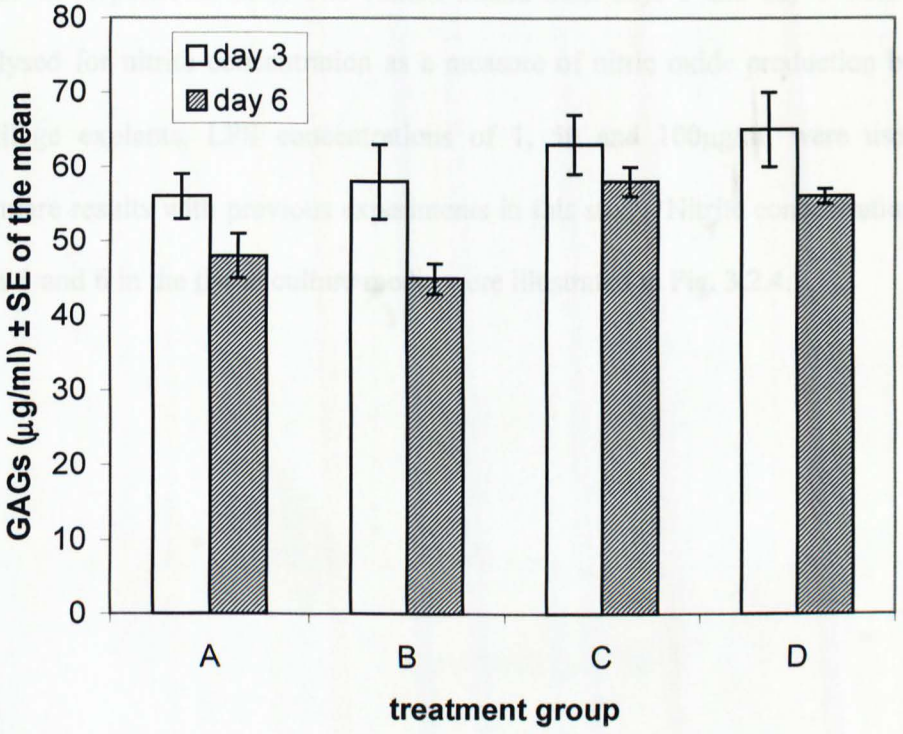


Fig. 3.2.3: Rat femoral head cartilage explants (n=8) were treated as follows: control (A) 1µg/ml LPS (B), 50µg/ml LPS (C) and 100µg/ml LPS (D) for a 6 day incubation. Culture media were changed on day 3. GAGs were measured in the culture media on days 3 and days 6. Analysis of data using a Students t test showed that there were no significant differences between control and treated groups.

GAG concentrations released into the tissue culture media were not changed by treatment with 1, 50 and 100µg/ml of LPS during 6 days of culture. As in experiments with IL-1β (see Fig. 3.1.4 and TNF-α (see Fig. 3.1.8), it was

observed that GAG concentrations in the media at day 6 were lower than GAG concentrations recorded at day 3 in all groups.

Stadler *et al.* (1991) showed that a monolayer of chondrocytes produced nitric oxide in response to LPS. The culture media from days 3 and day 6 were also analysed for nitrite concentration as a measure of nitric oxide production by rat cartilage explants. LPS concentrations of 1, 50 and 100μg/ml were used to compare results with previous experiments in this study. Nitrite concentrations at days 3 and 6 in the tissue culture media were illustrated in Fig. 3.2.4.

3.2.4: Effect of LPS on concentration of nitrite produced in the culture media by cartilage explants

Fig. 3.24: Effect of LPS on nitrite production by rat femoral head cartilage.

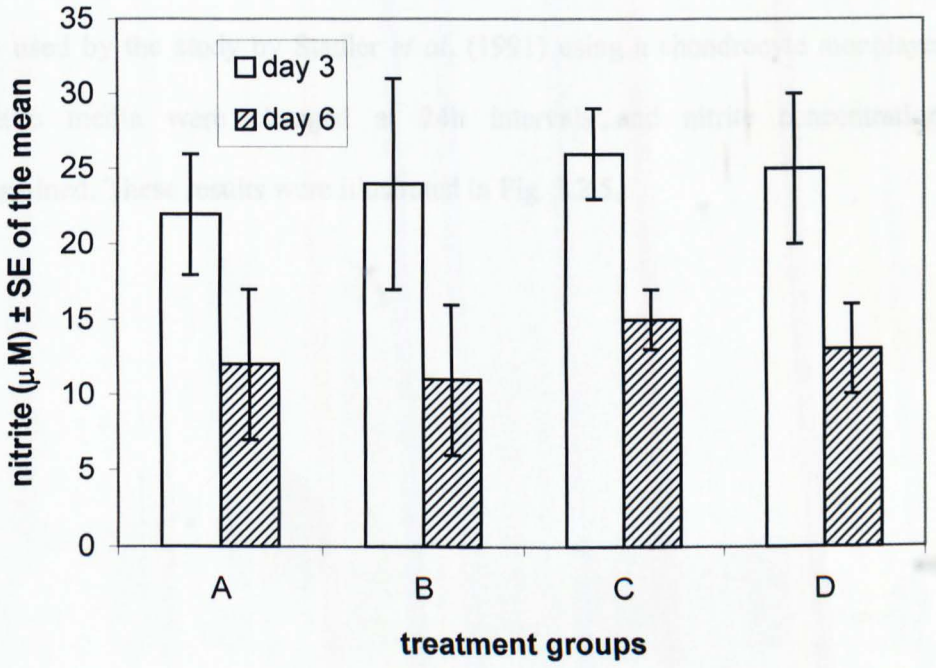


Fig. 3.24: Rat femoral head cartilage explants derived from (n=8) were treated with 1μg/ml – 100μg/ml of LPS for a 6 day incubation. Culture media was changed on day 3. Analysis of data using a Students t test showed that there was no significant differences observed between control groups and treated groups.

Observations in a study by Stadler *et al.*, (1991) showed that increased nitrite production was stimulated by LPS in a monolayer of chondrocytes. However, in this study the observed nitrite concentrations in culture media at day 3 and 6 were not significantly different from same day control groups.

The nitrite concentration generated in the culture media by rat cartilage on day 6 had diminished in all groups compared to nitrite concentrations detected on day 3. A time course of nitrite production was established to determine how the production of nitrite changed with time following treatment with 100μg/ml of LPS. This concentration was used to compare with the highest concentration of LPS used by the study by Stadler *et al.* (1991) using a chondrocyte monolayer. Culture media were changed at 24h intervals and nitrite concentrations determined. These results were illustrated in Fig. 3.2.5.

Fig. 3.2.5: Effect of LPS on concentration of nitrite produced in the culture media by cartilage explants during a time course of 6 days

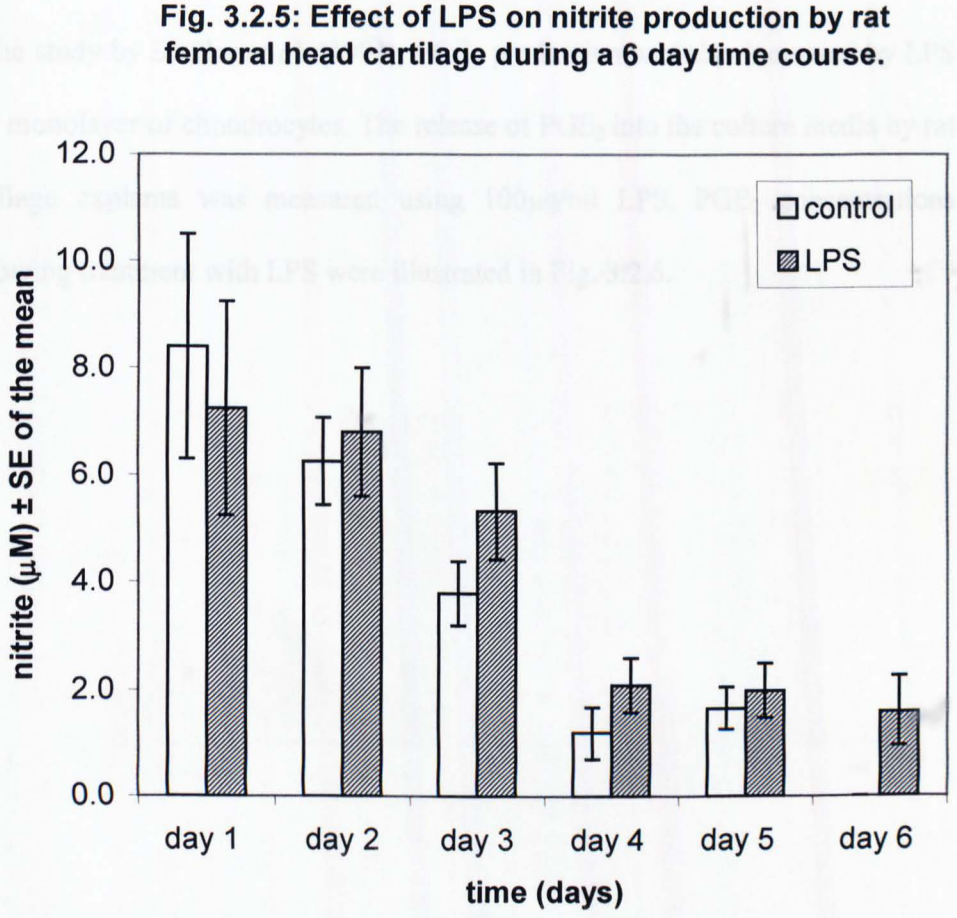


Fig. 3.2.5: A time course of nitrite production by rat femoral head cartilage in tissue culture supernatant was measured during a six day period at 24 hour intervals following treatment with 100 μg/ml LPS. Analysis of data using a Students t test showed that there were no significant differences between control and treated groups.

Nitrite produced in the group treated with 100μg/ml LPS showed no significant difference controls in this experiment (see Fig. 3.2.5). The concentration of nitrite

diminished in both control and 100μg/ml LPS groups during the time course of six days.

In the study by Stadler *et al.* (1991), PGE_2 production was also increased by LPS in a monolayer of chondrocytes. The release of PGE_2 into the culture media by rat cartilage explants was measured using 100μg/ml LPS. PGE_2 concentrations following treatment with LPS were illustrated in Fig. 3.2.6.

3.2.6: Effect of LPS on concentration of PGE_2 produced by cartilage explants

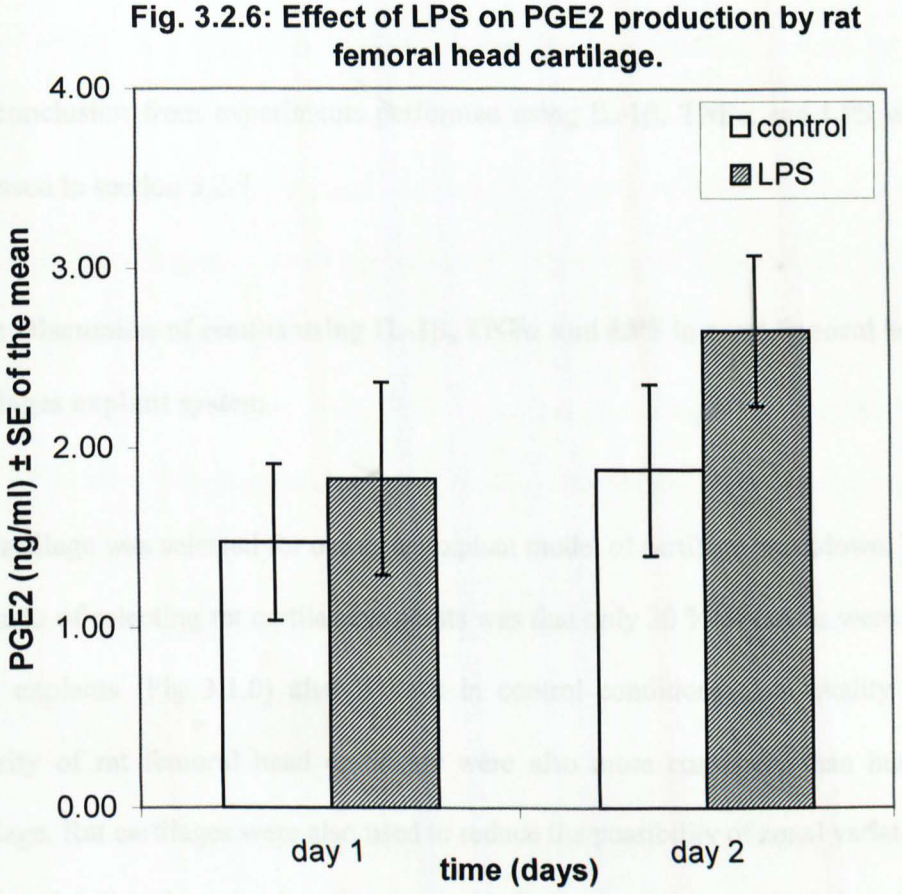


Fig. 3.2.6: Rat femoral head cartilage explants (n=8) were treated with 100μg/ml of LPS for a 48 hour incubation with a change of media at 24 hours. PGE_2 concentration were measured in the culture media and compared to controls. Analysis of data using a Students t test showed that there were no significant differences between control and treated groups.

PGE_2 concentrations were not significantly different in the 100μg/ml LPS treated group compared to the respective same day control groups (see Fig. 3.2.6). This result indicated that PGE2 concentrations were not increased by LPS,

contradicting the study performed by Stadler *et al.*, (1991) using a chondrocyte monolayer system.

The conclusion from experiments performed using IL-1β, TNFα and LPS were discussed in section 3.2.7

3.2.7: Discussion of results using IL-1β, TNFα and LPS in a rat femoral head cartilages explant system

Rat cartilage was selected for use as an explant model of cartilage breakdown. The rationale of selecting rat cartilage explants was that only 20 % of GAGs were lost from explants (Fig 3.1.0) after 6 days in control conditions. The quality and quantity of rat femoral head cartilages were also more consistent than human cartilage. Rat cartilages were also used to reduce the possibility of zonal variations (section 1.1.5) influencing the responses of individual cartilage explants in culture i.e. entire rat femoral head cartilages were used as cartilage explants.

The rat femoral head cartilage explant model was tested with three pro-inflammatory stimuli, IL-1β, TNF-α and LPS. These inflammatory mediators have all been implicated as mediators of cartilage breakdown in experimental models and have been shown to increase production of other markers of inflammation (Seed *et al.*, 1993; Hanglow *et al.*,1995; Stefanovic-Racic *et al.*, 1997; Bird *et. al.*, 1997; Sandy *et al.*, 1999; Spirito *et al.*, 1995 and Bottomley *et al.*, 1997).

In this study GAGs were measured as an indicator of cartilage breakdown. NO and PGE_2 production were quantified as indicators of chondrocyte response to inflammatory mediators. IL-1β, did not produce a significant change in GAG concentrations in cartilage explant digests (Fig. 3.1.2) or tissue culture media collected from explant cultures (Fig 3.1.3). This results contradicted studies suggesting that IL-1β produced increased loss of GAGs from cartilage explants (Seed *et al.*, 1993; Hanglow *et al.*, 1995; Spirito *et al.*, 1995; Stefanovic-Racic *et al.*, 1997). However, only the study by Seed *et al.*, (1993) utilised rat cartilage explants as a model of cartilage breakdown. The results obtained in this study supported research by Desa *et al.* (1989) that showed that IL-1 did not reduce GAG concentrations in rat cartilage explants compared to controls.

This varied result suggests that regulation of GAGs loss from cartilage explants may vary in different types of cartilage and in cartilage derived from different species. This species variability was subsequently confirmed in a study by Greenwald *et al.* (1998) that showed rats produce MMP-8, and MMP-13, but not MMP-1. It is therefore possible that rat cartilage therefore respond to treatment with IL-1β differentially from cartilage explants derived from other species. Despite these reports, it is possible to produce experimental models of arthritis in rats *in vivo* using models such as dimethyl dioctadecyl ammonium bromide (DDA) induced arthritis (Mia *et al.*, 2000).

IL-1β induced GAG loss from cartilage explants was not evident in the current

study, however, treatment with IL-1β produced a significant increase in production of both nitrite (Fig. 3.1.4) after 3 days and PGE_2 (Fig 3.1.6) after days 1 and 2 in the culture media. This supported previous studies that NO (Stefanovic-Racic *et al.* 1997; Bird *et. al.*, 1997; Blanco and Lotz, 1995; Berenbaum *et al.*, 1996) production were mediated by IL-1β in cartilage explant studies.

TNFα has been implicated as an important mediator of inflammation and cartilage breakdown (Homandberg *et al.*, 1998; Stichtenoth and Frolich, 1998), although it has not been directly linked to reduction of GAG concentrations in rat cartilage explant models. In this study TNFα did not affect GAG profiles in cartilage explants digests (Fig. 3.1.7) or in collected culture media (Fig 3.1.8). However, 100 ng/ml of TNFα produced a significant elevation in production of nitrite (Fig. 3.19) after 3 days in the culture media. This contradicted a report by Stadler *et al.*, (1991) that suggested lapine chondrocytes do not release nitric oxide in response to TNFα. However, a study by Badger *et al.*, (1998) showed that nitric oxide synthase is upregulated by TNFα in bovine chondrocytes. This suggests that chondrocytes derived from different species respond differentially to TNF-α. The method of using chondrocytes in a monolayer culture may also alter their responsiveness to inflammatory mediators such as TNFα and IL-1β.

In the present study it was shown that only the highest concentration (100 ng/ml) of TNFα (100 ng/ml) used resulted in increased production of nitrite and that TNF-α was less potent than IL-1β as a mediator of nitrite production at equivalent

concentrations. Unlike IL-1β, TNFα failed to influence the production of PGE_2 (Fig 3.2.1) by rat cartilage explants. These results were supported in a study performed by Berenbaum *et al.*, (1996) that showed chondrocyte monolayers do not produce PGE_2 in response to TNF-α treatment.

A study by Morales *et al.*, (1984) suggested LPS was a mediator of GAG loss from bovine cartilage explants. Stadler et al., (1991) produced a study that suggested LPS mediated production of both NO and PGE_2 from lapine chondrocyte monolayers. However, in the current study LPS did not significantly change GAG concentrations in cartilage explant digests (Fig. 3.2.2), GAG concentrations in media (Fig. 3.2.3), or production of nitrite (Fig 3.2.5) and PGE_2 (Fig. 3.2.6) by cartilage explants. These apparent deviations from the dogma that LPS mediates cartilage breakdown may have been a result of cartilage species variability in response to treatment with inflammatory mediators. Stadler, (1991) used lapine chondrocytes and Morales, (1984) used bovine explants as compared to this model where rat cartilage explants were used.

In this study, IL-1β proved to be the most potent inflammatory mediator with respect to increased production of nitrite and PGE_2. Since IL-1β increased release of nitrite and PGE_2 into the culture media from rat cartilage, it was selected as a positive control stimulus in the model to compare with the effects of CSFs on rat cartilage explants (see chapter 4).

Chapter 4:

Effects of colony stimulating factors on rat femoral head cartilage explants.

4.1.0 Introduction

Colony stimulating factors (CSFs) have been identified as inflammatory mediators that are produced by chondrocytes (Campbell *et al.*, 1991; Campbell *et al.*, 1993) and human synovial fibroblasts (Hamilton *et al.*, 1993). However, the effect of CSFs on cartilage has not been elucidated. The objective of this study was to determine if the CSFs regulated cartilage explants activity with respect to GAG content, nitrite and PGE_2 production. In the previous study (see chapter 3) the effects of IL-1β, TNF-α and LPS were measured with respect to regulation of GAG content and release by rat femoral head cartilage. The effects of IL-1β, TNF-α and LPS were also assessed with respect to PGE_2 and NO production. IL-1β was established as a positive control with respect to nitrite and PGE_2 production by cartilage explants. The effects of CSFs on regulation of GAGs, NO and PGE_2 in cartilage explants were measured and compared to IL-1β.

To determine if CSFs directly regulate chondrocyte activity, a series of experiments were performed to measure GAG content in cartilages following culture and loss of GAGs into the culture media at days 3 and 6 during a 6 day culture. Release of nitrite at days 3 and 6 and release of PGE_2 into the culture media at days 1 and 2 were also measured following treatment with CSFs. Since concentrations of CSFs produced by cartilage and surrounding tissues in the synovial joint have not been determined, a dose response was measured with each

G-CSF, GM-CSF, M-CSF and IL-3 at 1, 10 and 50 ng/ml. This provided data that were comparable with those obtained using equivalent concentrations of IL-1β (see chapter 3).

4.1.1 Effect of G-CSF on GAG concentration in post culture cartilage explants

Fig. 4.1.1: Effect of G-CSF on GAG content in rat femoral head cartilage

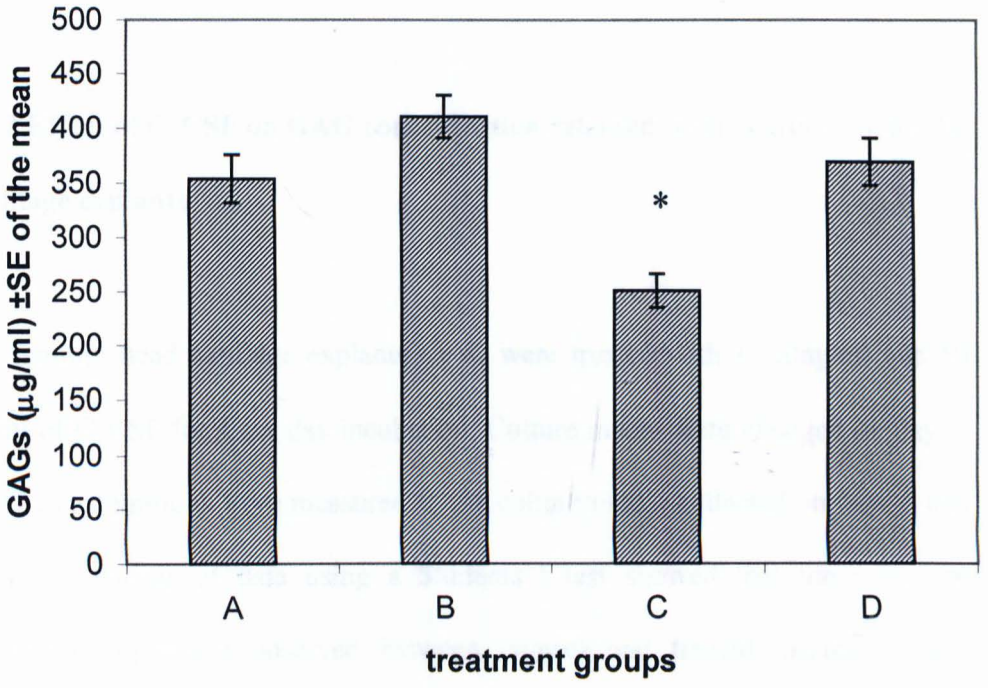


Fig. 4.1.1: Rat femoral head cartilage explants (n=8) were treated as follows: control (A), 1ng/ml G-CSF (B), 10ng/ml G-CSF (B) and 50 ng/ml G-CSF (C) for a six day incubation. Culture media were changed on day 3. GAGs concentrations were measured in the cartilage explant digests following the 6 day culture. Analysis of data using a Students t test showed showed that G-CSF at 10 ng/ml a produced a significant (*$P<0.05$) reduction in GAG concentrations in group C compared to the control (group A).

GAG concentrations in cartilages showed no significant difference compared to controls following treatment with 1ng/ml and 50ng/ml G-CSF for 6 days, however treatment with 10 ng/ml of G-CSF reduced levels of GAGs in the cartilage digests. GAG loss from cartilage explants was also measured in the culture media following treatment with 1, 10 and 50 ng/ml of G-CSF. This was measured on days 3 and 6 (see Fig. 4.1.2).

4.1.2 Effect of G-CSF on GAG concentration released in the culture media by cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 1, 10ng/ml and 50 ng/ml of G-CSF for a six day incubation. Culture media were changed on day 3. GAG concentrations were measured in the culture media collected on days 3 and days 6. Analysis of data using a Students t test showed that there was no significant difference observed between control and treated groups. It was observed that GAG concentrations in the media at day 6 were lower than GAG concentrations recorded at day 3 in all groups.

The culture media from days 3 and 6 were also analysed for nitrite concentration as a measure of nitric oxide production by rat cartilage explants. Nitrite concentrations at days 3 and 6 in the tissue culture media are illustrated in Fig. 4.1.3.

4.1.3: Effect of G-CSF on concentration of nitrite produced in the culture cartilage explants

Fig. 4.1.3 Effect of G-CSF on production of nitrite by rat femoral head cartilage

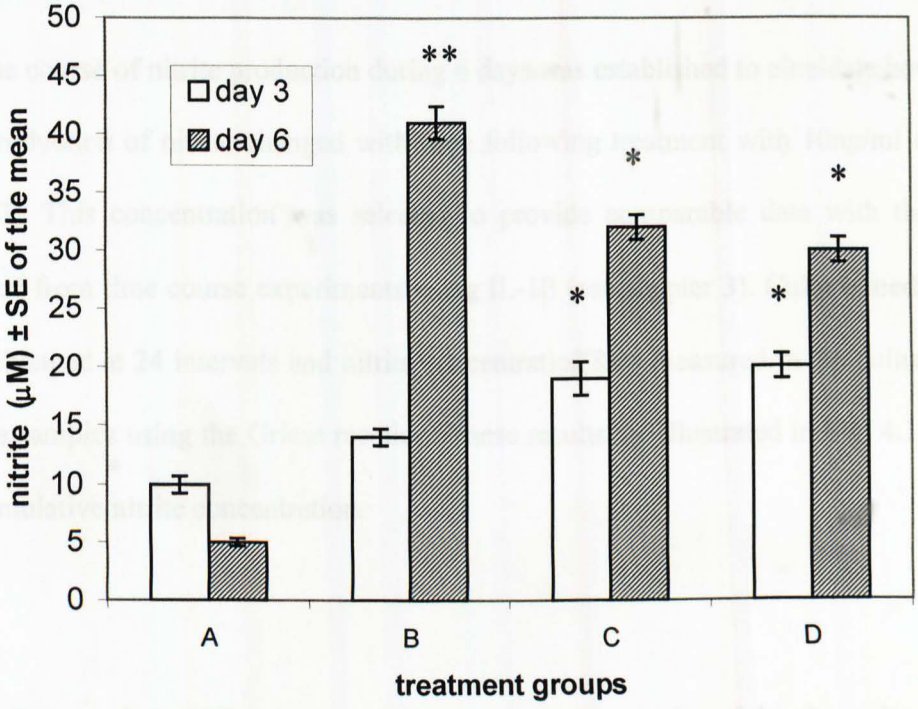


Fig. 4.1.3: Rat femoral head cartilage explants (n=8) were treated as follows: control (A), 1ng/ml (B) G-CSF, 10ng/ml G-CSF (C) and 50 ng/ml of G-CSF (D) for a six day incubation. Culture media were changed on day 3. Nitrite concentrations were measured in the culture media collected on days 3 and days 6. Analysis of data using a Students t test showed showed that G-CSF at 1 ng/ml caused a significant increase in nitrite concentration in groups C (*P<0.05) and D (*P<0.05) after 3 days compared to respective controls. G-CSF produced a significant increase in nitrite concentrations in group B (**P<0.01), C (*P<0.05) and D (*P<0.05) compared to the control (group A) after 6 days.

Nitrite concentrations in culture media were significantly increased by 10 ng/ml ($p<0.05$) and 50 ng/ml IL-1β ($p<0.05$) compared to same day controls after 3 days. Nitrite concentrations in culture media at day 6 were significantly increased by 1ng/ml ($p<0.01$), 10ng/ml G-CSF ($p<0.05$) and 50ng/ml ($p<0.05$) compared to respective same day controls.

A time course of nitrite production during 6 days was established to elucidate how the production of nitrite changed with time following treatment with 10ng/ml of G-CSF. This concentration was selected to provide comparable data with that derived from time course experiments using IL-1β (see chapter 3). Culture media was changed at 24 intervals and nitrite concentratio2Tas measured in the culture media samples using the Griess reaction. These results are illustrated in Fig. 4.1.4 as cumulative nitrite concentration.

4.1.4 Effect of G-CSF on concentration of nitrite produced in the culture media by cartilage explants during a time course of 6 days

A time course of nitrite concentrations produced by rat femoral head cartilages (n=8) in tissue culture media were measured during a six day period at 24 hour intervals following stimulation with 10ng/ml G-CSF. Analysis of data using a Students t test showed that there was no significant difference was produced in the 10ng/ml G-CSF group compared to respective control groups.

PGE_2 concentrations were also measured in the culture media following treatment with 10 ng/ml G-CSF. This concentration and duration of culture was selected to allow direct comparison with equivalent IL-1β data obtained (see chapter 3). PGE_2 concentrations were measured in the culture media at days 1 and 2 following treatment with G-CSF. PGE_2 concentrations were illustrated in Fig. 4.1.5.

4.1.5: Effect of G-CSF on concentration of PGE_2 produced by cartilage explants

Fig. 4.1.5: Effect of G-CSF on PGE2 production by rat femoral head cartilage

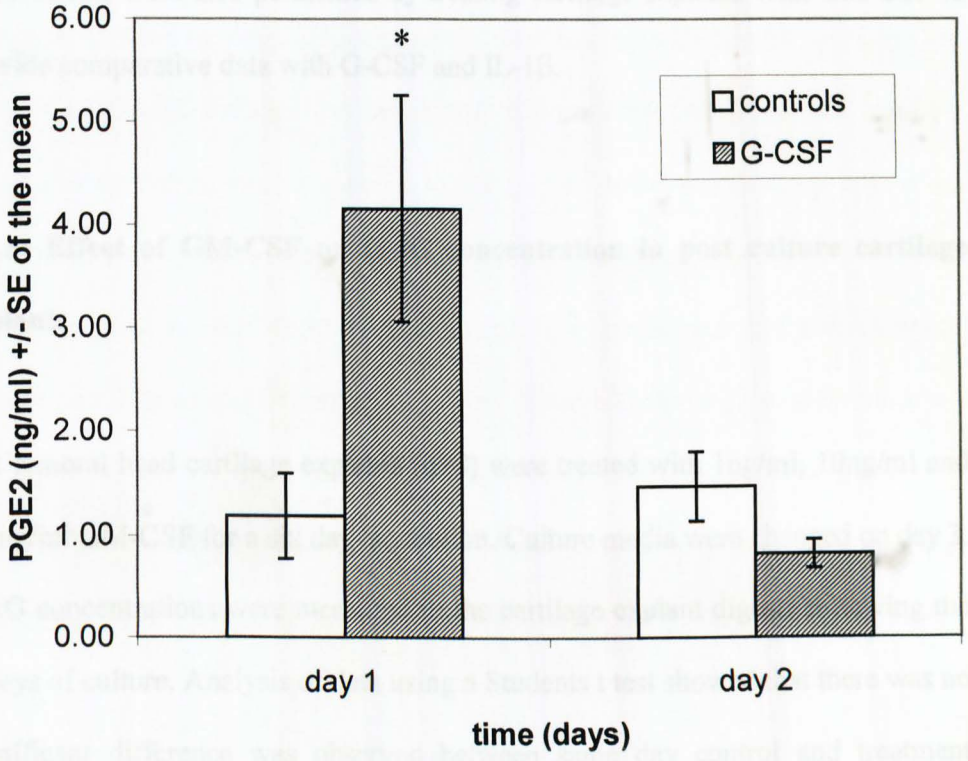


Fig. 4.1.5: Rat femoral head cartilage explants (n=8) were treated with 10ng/ml G-CSF for a 48 hour incubation with a change of media at 24 hours. PGE_2 concentrations were measured in the culture media and compared to controls. Analysis of data using a Students t test showed showed that G-CSF at 10 ng/ml produced significant increase ($p<0.05$) on day 1 compared to the same day control group.

The group of cartilage explants treated with 10 ng/ml G-CSF showed a significant ($p<0.05$) increase in concentration of PGE_2 in the culture media after day 1 compared to the same day control group. There was no significant difference observed between the 10ng/ml G-CSF group and control group at 48 hours. Experiments were also performed by treating cartilage explants with GM-CSF to provide comparative data with G-CSF and IL-1β.

4.1.6: Effect of GM-CSF on GAG concentration in post culture cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 1ng/ml, 10ng/ml and 50 ng/ml GM-CSF for a six day incubation. Culture media were changed on day 3. GAG concentrations were measured in the cartilage explant digests following the 6 days of culture. Analysis of data using a Students t test showed that there was no significant difference was observed between same day control and treatment groups.

The GAG loss from cartilage explants was also measured in the culture media collected following treatment with 1, 10 and 50ng/ml of GM-CSF. This was measured following days 3 and 6 of culture.

4.1.7 Effect of GM-CSF on GAG concentration released in the culture media by cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 1ng/ml, 10ng/ml and 50 ng/ml GM-CSF for a six day incubation. Culture media were changed on day 3. GAG concentrations were measured in the culture media collected on days 3 and days 6. As with G-CSF, analysis of data using a Students T test showed that there was no significant difference was observed between control and and treatment groups. As in previous experiments, it was observed that GAG concentrations in the media at day 6 were lower than GAG concentrations recorded at day 3 in all groups. The culture media from days 3 and 6 were also analysed for nitrite concentration as a measure of nitric oxide production by treatment with GM-CSF.

4.1.8: Effect of GM-CSF on concentration of nitrite produced in the culture media by cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 1ng/ml, 10ng/ml and 50 ng/ml GM-CSF for a six day incubation. Culture media were changed on day 3. Nitrite concentrations were measured in the culture media collected on days 3 and days 6. However, unlike treatment with G-CSF, analysis of data using a Students t test showed that there was no significant difference was observed between

GM-CSF treated group and same day control. As in previous experiments, nitrite generated in the culture media by rat cartilage diminished between day 3 and day 6 in both control and treated groups.

PGE_2 concentrations were measured in the culture media following treatment with 10 ng/ml GM-CSF. PGE_2 concentrations were measured in the culture media at days 1 and 2. This concentration and duration of culture were selected to provide comparative data with that obtained from previous experiments with IL-1β and G-CSF data.

4.1.9: Effect of GM-CSF on concentration of PGE_2 produced by cartilage explants

There was no significant difference observed between the 10ng/ml GM-CSF treated cartilage explants and control group. PGE_2 concentrations in collected culture media after days 1 and 2. Experiments were then performed by treating cartilage explants with M-CSF to provide comparative data with G-CSF, GM-CSF and IL-1β.

4.2.0: Effect of M-CSF on GAG concentration in post culture cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 1ng/ml, 10ng/ml and 50 ng/ml M-CSF for a six day incubation. Culture media were changed on day 3. GAG concentrations were measured in the cartilage explant digests following the 6 day culture. Analysis of data using a Students t test showed that there was no significant difference was observed between the control and treatment groups. The GAG loss from cartilage explants was also measured in the culture media on day 3 and 6 following treatment with 1, 10 and 50ng/ml of M-CSF.

4.2.1: Effect of M-CSF on GAG concentration released in the culture media by cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 1ng/ml, 10ng/ml and 50 ng/ml M-CSF for a six day incubation. Culture media were changed on day 3. GAG concentrations were measured in the culture media collected on day 3 and 6. Analysis of data using a Students t test showed that there was no significant difference was observed between the control and treated groups. It was observed that GAG concentrations in the media at day 6 were lower than GAG concentrations recorded at day 3 in both control and treatment groups. The culture media from day 3 and 6 were also analysed for nitrite concentration as a measure of nitric oxide production mediated by M-CSF.

4.2.2: Effect of M-CSF on concentration of nitrite produced in the culture media by cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 1ng/ml, 10ng/ml and 50 ng/ml M-CSF for a six day incubation. Culture media were changed on day 3. Nitrite concentrations were measured in the culture media collected on day 3 and 6. Analysis of data using a Students t test showed that there was no significant difference observed between the control and treatment groups. The nitrite generated in the culture media by rat cartilage diminished between day 3 and 6. PGE_2 concentrations were measured in the culture media at days 1 and 2 following treatment with 10 ng/ml M-CSF.

4.2.3: Effect of M-CSF on concentration of PGE_2 produced by cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 10ng/ml of M-CSF for a 2 day incubation with a change of media at day 1. PGE_2 concentrations were measured in the culture media and compared to controls. Analysis of data using a Students t test showed that there was no significant difference observed between the control and treatment group. Experiments were then performed by treating cartilage explants with IL-3 to provide comparative data with G-CSF, GM-CSF, M-CSF and IL-1β.

4.2.4: Effect of IL-3 on GAG concentration in post culture cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 1ng/ml, 10ng/ml and 50 ng/ml IL-3 for a six day incubation period. Culture media were changed on day 3. GAG concentrations were measured in the cartilage explant digests following the 6 day culture. Analysis of data using a Students t test showed that there was no significant difference observed between control and treatment groups. GAG loss from cartilage explants was also measured in the culture media following treatment with 1, 10 and 50ng/ml IL-3.

4.2.5: Effect of IL-3 on GAG concentration released in the culture media by cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 1ng/ml, 10ng/ml and 50 ng/ml IL-3 for a six day incubation. Culture media were changed on day 3. GAG concentrations were measured in the culture media collected on day 3 and 6. Analysis of data using a Students t test showed that there was no significant difference was observed between control and and treatment groups. As in previous experiments, it was observed that GAG concentrations in the media at day 6 were lower than GAG concentrations recorded at day 3 in all groups. The culture media from day 3 and 6 were also analysed for nitrite concentration as a measure of nitric oxide production mediated by IL-3.

4.2.6: Effect of IL-3 on concentration of nitrite produced in the culture media by cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 1ng/ml, 10ng/ml and 50 ng/ml IL-3 for a six day incubation. Culture media were changed on day 3. Nitrite concentrations were measured in the culture media on day 3 and 6. Analysis of data using a Students t test showed that there was no significant difference observed between control and treated groups. The nitrite generated in the culture media by rat cartilage diminished between day 3 and 6. PGE_2 concentrations were also measured in the culture media following treatment with 10 ng/ml IL-3. PGE_2 concentrations were measured in the culture media at day 1 and 2 and are illustrated in Fig 4.2.6.

4.2.6: Effect of IL-3 on concentration of PGE_2 produced by cartilage explants

Fig. 4.2.6: Effect of IL-3 on PGE2 production by rat femoral head cartilage

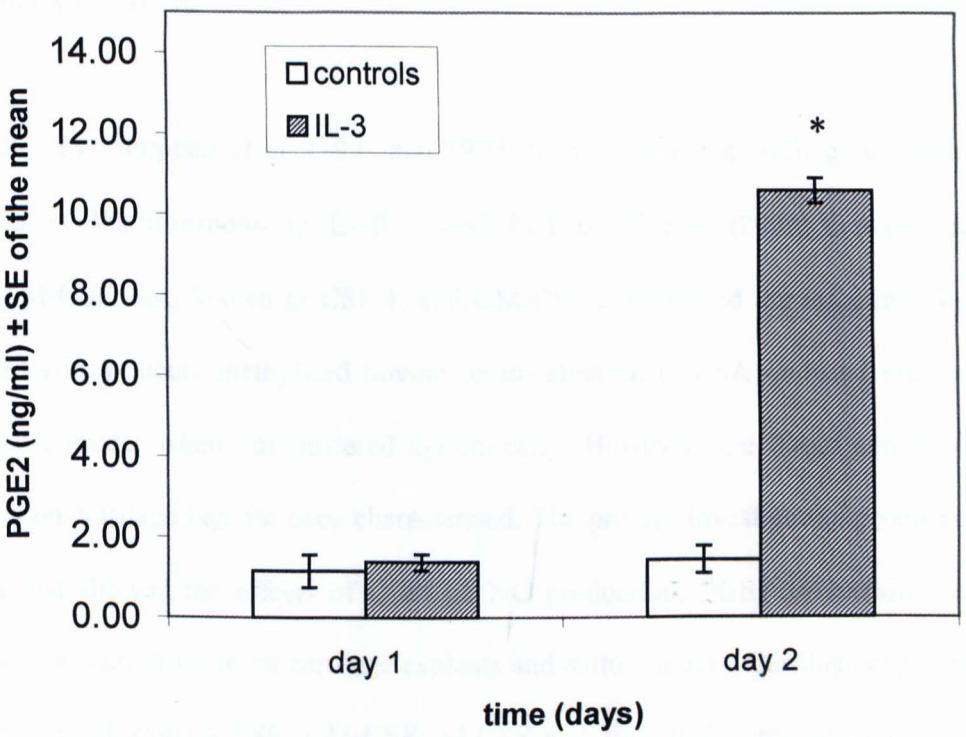


Fig. 4.2.6: Rat femoral head cartilage explants (n=8) were treated with 10ng/ml of IL-3 for a 48 hour hour incubation with a change of media at 24 hours. PGE_2 concentrations were measured in the culture media and compared to controls. Analysis of data using a Students t test showed showed that IL-3 at 10 ng/ml a produced significant increase ($p<0.05$) on day 2 compared to the same day control group.

There was a significant increase ($p<0.05$) in concentrations of PGE_2 in the group treated with IL-3 compared to the same day control group following day 2 in

culture. However, there was no significant difference observed between control and treatment groups following day 1 of culture.

4.2.7: Discussion comparing effects of CSFs on rat femoral head cartilage compared to IL-1β

Studies by Campbell *et al.* (1991 and 1993) have shown that cartilage explants produce CSFs in response to IL-1β. A study by Bischof *et al.*, (2000) showed that both M-CSF, also known as CSF-1, and GM-CSF exacerbated the inflammatory arthritis in an acute methylated bovine serum albumin (mBSA)-induced murine arthritis model when administered systemically. However, the direct action of CSFs on cartilage has not been characterised. The present investigation produced data that showed the effects of CSFs on NO production, PGE_2 production and GAG concentrations in rat cartilage explants and culture media. Cartilage explants were treated with G-CSF, GM-CSF, M-CSF and IL-3 and compared with the effects of IL-1β (see chapter 3).

Treatment of cartilages with G-CSF at concentrations of 1, 10 and 50 ng/ml caused a significant increase in both nitrite (Fig. 4.1.1) and PGE_2 (Fig. 4.1.3) concentrations produced by rat cartilage explants. G-CSF also produced a significant ($p<0.05$) reduction in GAG concentrations (Fig. 4.1.5) detected in rat cartilage explant digests. Both G-CSF and IL-1β increased nitrite and PGE_2 levels, though unlike G-CSF, IL-1β did not reduce GAG levels in cartilage explant

digests. However, despite the reduction of GAG concentrations in explants treated with G-CSF, GAGs were not elevated in the culture media following treatment with G-CSF. Since GAGs were not lost from G-CSF explants, it was speculated that G-CSF may have reduced GAG synthesis or GAG incorporation into the cartilage matrix.

Unlike G-CSF, GM-CSF and M-CSF did not cause a change in production of nitrite, PGE_2 or GAG levels following administration to cartilage explants. However IL-3, also known as multi-CSF, produced a significant increase in concentrations of PGE_2 produced in the culture media by rat cartilage explants.

Since studies by Campbell *et al.* (1991 and 1993) suggested that IL-1β caused production of CSFs by cartilage explants and chondrocytes, it was rationalised that CSFs and IL-1β may be localised around cartilage and chondrocytes when considering an *in vivo* inflammatory response. Therefore, effects of combining treatments of CSFs with IL-1β on rat cartilage explants were examined in the following study (Chapter 5).

Chapter 5:

The effects of CSFs combined with IL-1β on rat femoral head cartilage explants.

5.1.0: Introduction

The results of the previous investigation (chapter 3) demonstrated that IL-1β is the most potent inflammatory mediator with respect to nitrite and PGE_2 production by rat femoral head cartilage explants, compared to TNF-α and LPS. The results displayed in chapter 4 showed that G-CSF also increased production of nitrite and PGE_2 by rat cartilage explants and G-CSF also influenced GAG concentrations in cartilage explant digests. Treatment of cartilage explants with IL-3 caused an increased production of PGE_2. Campbell *et al.* (1991 and 1993) showed that IL-1β caused production of CSFs by cartilage explants and chondrocytes and Bischof *et al.* (2000) showed that CSF may influence an experimental murine model of arthritis. Therefore, a study was performed to examine the combined effects of IL-1β with CSFs on the rat cartilage explant model system.

A study was performed by combining treatments of CSFs with IL-1β to determine if the combined effect of the CSFs with IL-1β was greater or less than individual effects of these cytokines in rat cartilage explants. As in previous investigations, GAG concentrations in cartilage explant digests and culture media were determined and concentrations of nitrite and PGE_2 released into the culture media were also measured.

In the present study IL-1β used were used at a concentration of 10 ng/ml to provide comparative data with those illustrated in chapters 3 and 4. CSFs were

used at a concentration of 10ng/ml since G-CSF and IL-3 were demonstrated to have regulatory effects on cartilage at these concentrations (see chapter 4). GAG concentrations in cartilage explant digests were determined, as previously described following a 6 day culture period with a change of culture media at 24 hour intervals. Media samples were collected at 24 hours intervals and analysed to determine a time course of nitrite production during 6 days. GAG concentrations were measured the culture media from days 1 and 2 in this study. This provided data that could be compared with both nitrite and PGE_2 concentrations in sample media collected on days 1 and 2. These parameters were quantified following combined treatments with G-CSF and IL-1β (see 5.1.1 - 5.1.4).

5.1.1: Effect of G-CSF and IL-1β on GAG concentration in post culture cartilage explants

Fig. 5.1.1: Effect of G-CSF and IL-1β on GAG content in rat femoral head cartilage

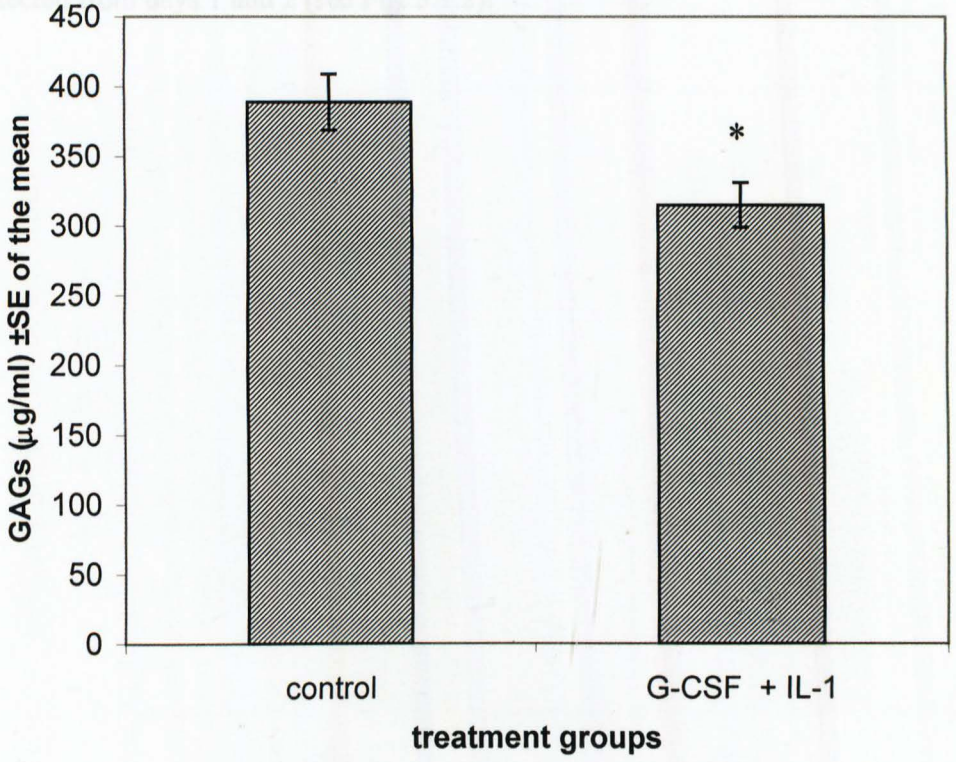


Fig. 5.1.1: Rat femoral head cartilage explants (n=8) were treated with 10ng/ml G-CSF and 10 ng/ml IL-1β for a six day incubation. Culture media were collected at 24 hour intervals. GAG concentrations were measured in rat cartilage explant digests following the 6 day culture. Analysis of data using a Students t test showed showed that G-CSF and IL-1β produced a significant (*= $P<0.05$) reduction in GAG concentrations compared to the control group.

GAG concentrations in cartilages were significantly reduced ($p<0.05$) following treatment with G-CSF and IL-1β for 6 days compared to the control group. This

effect was similar to that observed following treatment with G-CSF alone (see Fig. 4.11)., the combined effect of G-CSF and IL-1β was comparable to that of G-CSF alone. GAG loss from cartilage explants was also measured in the culture media collected following treatment with G-CSF and IL-1β. This was measured in media collected from days 1 and 2 (see Fig. 5.1.2).

5.1.2: Effect of G-CSF and IL-1β on GAG concentration released in the culture media by cartilage explants

Fig. 5.1.2: Effect of G-CSF and IL-1β on GAG release by rat femoral head cartilage

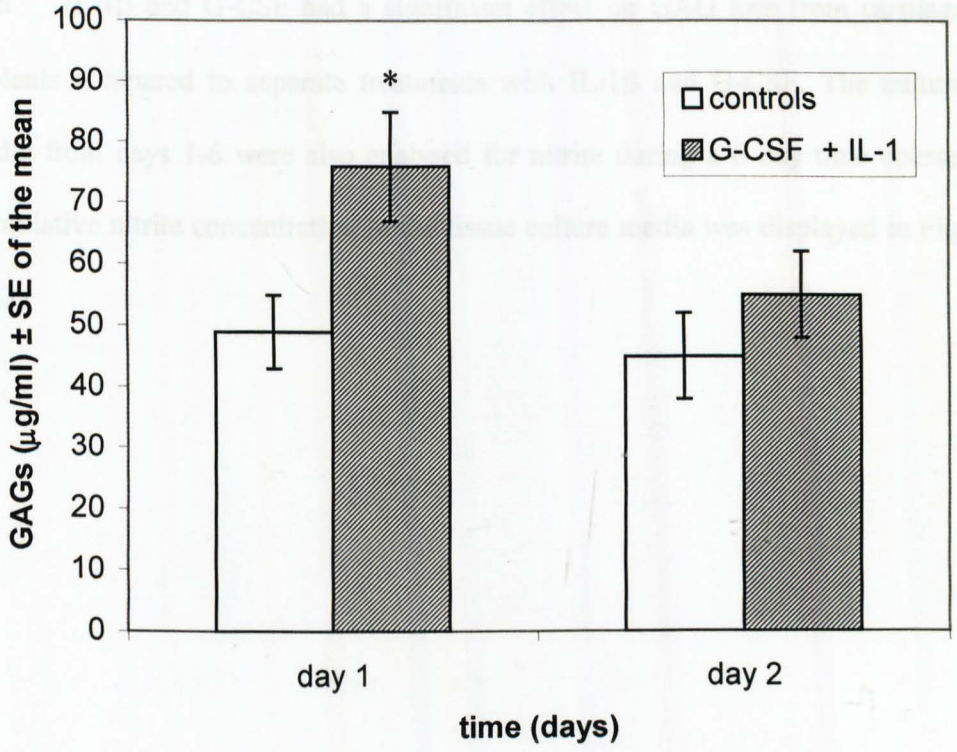


Fig. 5.1.2: Rat femoral head cartilage explants (n=8) were treated with 10ng/ml G-CSF and 10 ng/ml IL-1β for a six day incubation period. GAGs were measured in the culture media collected on days 1 and days 2. Analysis of data using a Students t test showed that G-CSF and IL-1β caused a significant (*$P<0.05$) increase in GAG concentrations in the media on day 1 compared to the same day control group.

GAG concentrations in the tissue culture media were significantly increased on day 1 following treatment with G-CSF and IL-1β during 6 days of culture. This is compared to media samples collected after 3 and 6 days from individual treatments with IL-1β and G-CSF that did not cause elevated concentrations of GAGs in media compared to controls. This demonstrated that combined treatment with IL-1β and G-CSF had a significant effect on GAG loss from cartilage explants compared to separate treatments with IL-1β and G-CSF. The culture media from days 1-6 were also analysed for nitrite during a 6 day time course. Cumulative nitrite concentration in the tissue culture media was displayed in Fig. 5.1.3.

5.1.3: Effect of G-CSF and IL-1β on cumulative concentration of nitrite produced in the culture media by cartilage explants during a time course of 6 days

Fig. 5.1.3: Effect of G-CSF and IL-1β on nitrite production by rat femoral head cartilage during a 6 day time course.

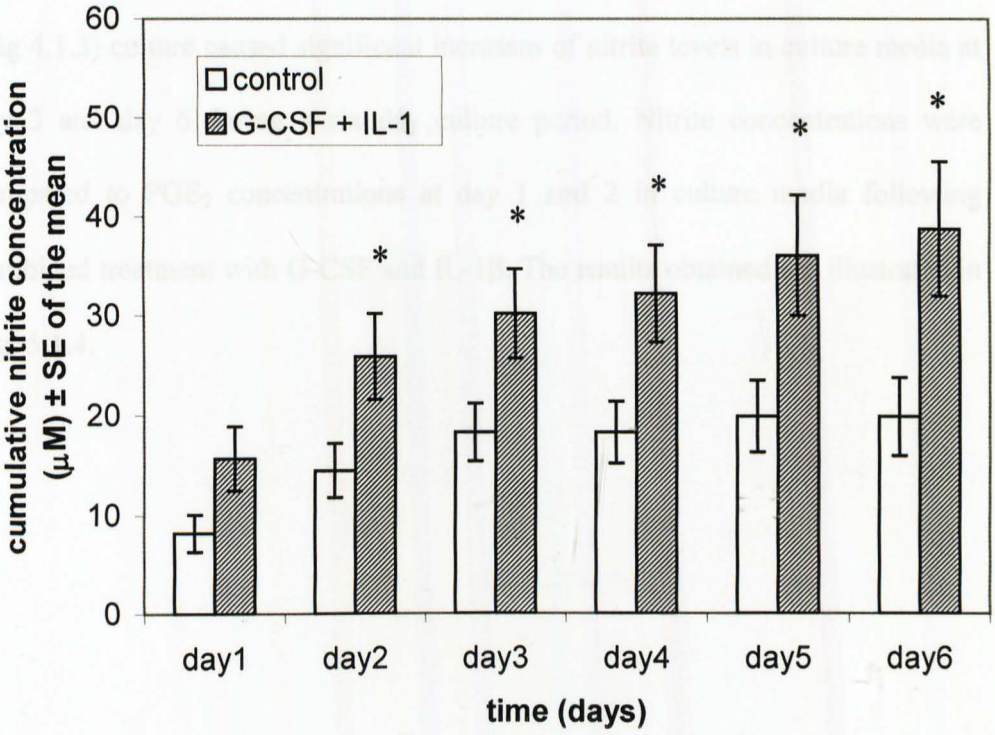


Fig. 5.1.3: A time course of cumulative nitrite production by rat femoral head cartilage explants (n=8) in tissue culture media was measured during a six day period at 24 hour intervals following stimulation with 10 ng/ml of G-CSF and 10 ng/ml of IL-1β. Analysis of data using a Students t test showed that G-CSF and IL-1β caused a significant (*$p<0.05$) increase in nitrite concentrations in the media on days 2, 3, 4, 5 and 6 compared to the same day control group.

Nitrite concentrations in culture media were significantly increased by combined treatment with G-CSF and IL-1β ($p<0.05$) compared to same day controls on days 2, 3, 4, 5 and 6. In previous experiments, individual treatments with IL-1β (Fig. 3.1.5) and G-CSF (section 4.1.4) did not cause a significant increase in concentrations of nitrite measured in media at 24 hour intevals during a 6 day time course. However, individual treatments with IL-1β (Fig. 3.1.4) and G-CSF (Fig 4.1.3) culture caused significant increases of nitrite levels in culture media at day 3 and day 6 during a six day culture period. Nitrite concentrations were compared to PGE_2 concentrations at day 1 and 2 in culture media following combined treatment with G-CSF and IL-1β. The results obtained are illustrated in Fig. 5.1.4.

5.1.4: Effect of G-CSF and IL-1β on concentration of PGE_2 produced by cartilage explants

Fig. 5.1.4: Effect of G-CSF and IL-1β on PGE2 production by rat femoral head cartilage

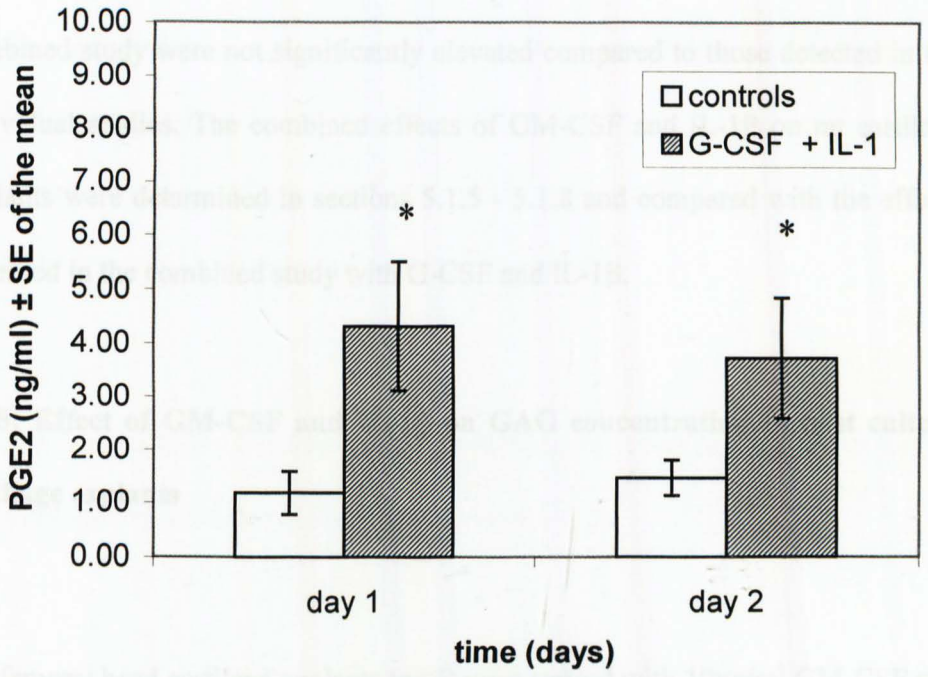


Fig. 5.1.4: Rat femoral head cartilage explants (n=8) were treated with 10ng/ml of G-CSF and 10 ng/ml IL-1β for a 48 hour incubation period with a change of culture media at 24 hours. PGE_2 concentrations were measured in the culture media on days 1 and 2. Analysis of data using a Students t test showed that G-CSF and IL-1β caused a significant (*$P<0.05$) increase in PGE_2 concentrations in the media on days 1 and 2 compared to the same day control group.

The group of cartilage explants treated with G-CSF and IL-1β showed a significant ($p<0.05$) increase in concentration of PGE_2 in the culture media after

day 1 and day 2 compared to the same day control group. In previous experiments, individual treatments with IL-1β (Fig. 3.1.6) and G-CSF (Fig 4.1.5) caused a significant increase in concentrations of PGE_2 measured in media samples at days 1 and 2. Treatment with both G-CSF and IL-1β resulted in a significant increases in PGE_2 concentrations on days 1 and 2, however, PGE2 concentrations in the combined study were not significantly elevated compared to those detected in the individual studies. The combined effects of GM-CSF and IL-1β on rat cartilage explants were determined in sections 5.1.5 - 5.1.8 and compared with the effects observed in the combined study with G-CSF and IL-1β.

5.1.5: Effect of GM-CSF and IL-1β on GAG concentration in post culture cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 10ng/ml GM-CSF and 10 ng/ml IL-1β for a six day incubation. Culture media were changed at 24 hour intervals. GAG concentrations were measured in rat cartilage explant digests following the 6 day culture. Analysis of data using a Students t test showed that there was no significant difference between the control and treatment groups. GAG concentrations were also measured in culture media collected on days 1 and 2.

5.1.6 Effect of GM-CSF and IL-1β on GAG concentration released in the culture media by cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 10ng/ml GM-CSF and 10 ng/ml IL-1β for a six day incubation. GAGs concentrations were measured in culture media collected on days 1 and days 2. Analysis of data using a Students t test showed that there was no significant difference observed between the control and treatment groups. Nitrite concentrations in culture media collected at 24 hour intervals in a 6 day culture period were also measured.

5.1.7: Effect of GM-CSF and IL-1β on nitrite produced in the culture media by cartilage explants during a time course of 6 days

A time course of nitrite production by rat femoral head cartilage (n=8) in tissue culture supernatant was measured during a six day period at 24 hour intervals following stimulation with 10 ng/ml of GM-CSF and 10 ng/ml of IL-1β. Analysis of data using a Students t test showed that there was no significant difference between the control and and treatment groups. PGE_2 concentrations were measured in culture media at day 1 and 2 following combined treatment with GM-CSF and IL-1β and compared with nitrite and GAG data.

5.1.8: Effect of GM-CSF and IL-1β on concentration of PGE_2 produced by cartilage explants

Fig 5.1.8: Effect of GM-CSF and IL-1β on PGE2 production by rat femoral head cartilage

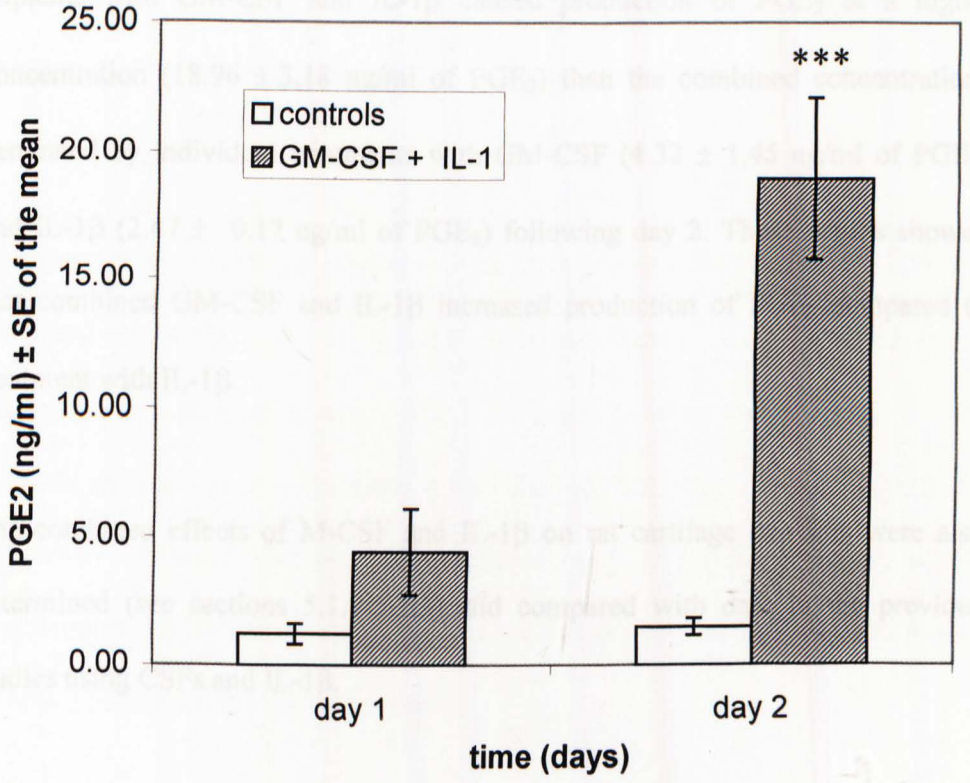


Fig. 5.1.8: Rat femoral head cartilage explants (n=8) were treated with 10 ng/ml of GM-CSF and 10 ng/ml IL-1β for a 48 hour incubation period with a change of media at 24 hours. PGE_2 concentrations were measured in the culture media and compared to controls. Analysis of data using a Students t test showed that GM-CSF and IL-1β caused a significant (***$P<0.001$) increase in PGE_2 concentrations in the media on day 2 compared to the same day control group.

The group of cartilage explants treated with GM-CSF and IL-1β showed a significant ($p<0.001$) increase in concentration of PGE_2 in the culture media after

day 2 compared to the same day control group. In a previous study, treatment with IL-1β (Fig 3.1.6) resulted in a significant increase in PGE_2 concentrations produced by rat cartilage explants in culture media. However, GM-CSF did not increase production of PGE_2 (see section 4.1.9). However, combined treatment of explants with GM-CSF and IL-1β caused production of PGE_2 at a higher concentration (18.96 ± 3.18 ng/ml of PGE_2) than the combined concentrations generated by individual treatments with GM-CSF (4.32 ± 1.45 ng/ml of PGE_2) and IL-1β (2.47 ± 0.17 ng/ml of PGE_2) following day 2. These results showed that combined GM-CSF and IL-1β increased production of PGE_2 compared to treatment with IL-1β.

The combined effects of M-CSF and IL-1β on rat cartilage explants were also determined (see sections 5.1.9-5.2.3) and compared with data in the previous studies using CSFs and IL-1β.

5.1.9: Effect of M-CSF and IL-1β on GAG concentration in post culture cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 10ng/ml M-CSF and 10 ng/ml IL-1β for a six day incubation period. Culture media were changed at 24 hour intervals. GAG concentrations were measured in rat cartilage explant digests following the 6 day culture. Analysis of data using a Students t test showed that there was no significant difference between the control and treatment groups.

GAG concentrations were also measured in culture media collected on days 1 and 2.

5.2.0: Effect of M-CSF and IL-1β on GAG concentration released in the culture media by cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 10ng/ml M-CSF and 10 ng/ml IL-1β for a six day incubation period. GAGs were measured in the culture media collected on days 1 and 2. As in the previous study using GM-CSF, analysis of data using a Students t test showed that there was no significant difference observed between the control and treatment groups. Nitrite concentrations were also measured in culture media samples collected at 24 hour intervals during a 6 day culture period.

5.2.1: Effect of M-CSF and IL-1β on nitrite produced in the culture media by cartilage explants during a time course of 6 days

A time course of nitrite production by rat femoral head cartilages (n=8) in tissue culture supernatant was measured during a six day period at 24 hour intervals following stimulation with 10 ng/ml of M-CSF and 10 ng/ml of IL-1β. As in the previous study using GM-CSF, analysis of data using a Students t test showed that

there was no significant difference observed between the control and and treatment groups. PGE_2 concentrations were also quantified in culture media at days 1 and 2 following combined treatment with M-CSF and IL-1β.

5.2.2: Effect of M-CSF and IL-1β on of PGE_2 produced by cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 10ng/ml of M-CSF and 10 ng/ml IL-1β for a 2 day period with a change of media at 24 hours. PGE_2 concentration was measured in the culture media and compared to control groups. Analysis of data using a Students t test showed that there was no significant difference between the control group and treatment groups.

The combined effects of IL-3 and IL-1β on rat cartilage explants were determined in sections 5.2.3-5.2.7 and compared with data in the previous combined studies using CSFs and IL-1β.

5.2.3: Effect of IL-3 and IL-1β on GAG concentration in post culture cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 10ng/ml IL-3 and 10 ng/ml IL-1β for a six day incubation period. Culture media were changed at 24 hour intervals. GAG concentrations were measured in rat cartilage explant digests following the 6 day culture. Analysis of data using a Students t test showed that there was no significant difference between the control and and treatment groups. GAG concentrations were also measured in culture media collected on days 1 and 2.

5.2.4: Effect of IL-3 and IL-1β on GAG concentration released into the culture media by cartilage explants

Rat femoral head cartilage explants (n=8) were treated with 10ng/ml IL-3 and 10 ng/ml IL-1β for a six day incubation period. GAG concentrations were measured in the culture media collected on days 1 and 2. Analysis of data using a Students t test showed that there was no difference between the control and treatment groups. Nitrite concentrations were also measured in culture media samples collected at 24 hour intervals during a 6 day culture period.

5.2.5: Effect of IL-3 and IL-1β on concentration of nitrite produced in the culture media by cartilage explants during a time course of 6 days

A time course of nitrite production by rat femoral head cartilage (n=8) in tissue culture supernatant was measured during a six day period at 24 hour intervals following stimulation with 10 ng/ml of IL-3 and 10 ng/ml of IL-1β. Analysis of data using a Students t test showed that there was no significant difference was observed between control and treatment groups. PGE_2 concentrations were also quantified in culture media at day 1 and 2 following combined treatment with IL-3 and IL-1β.

5.2.6: Effect of IL-3 on concentration of PGE_2 produced by cartilage explants

Fig. 5.2.6: Effect of IL-3 and IL-1β on PGE2 production in rat femoral head cartilage

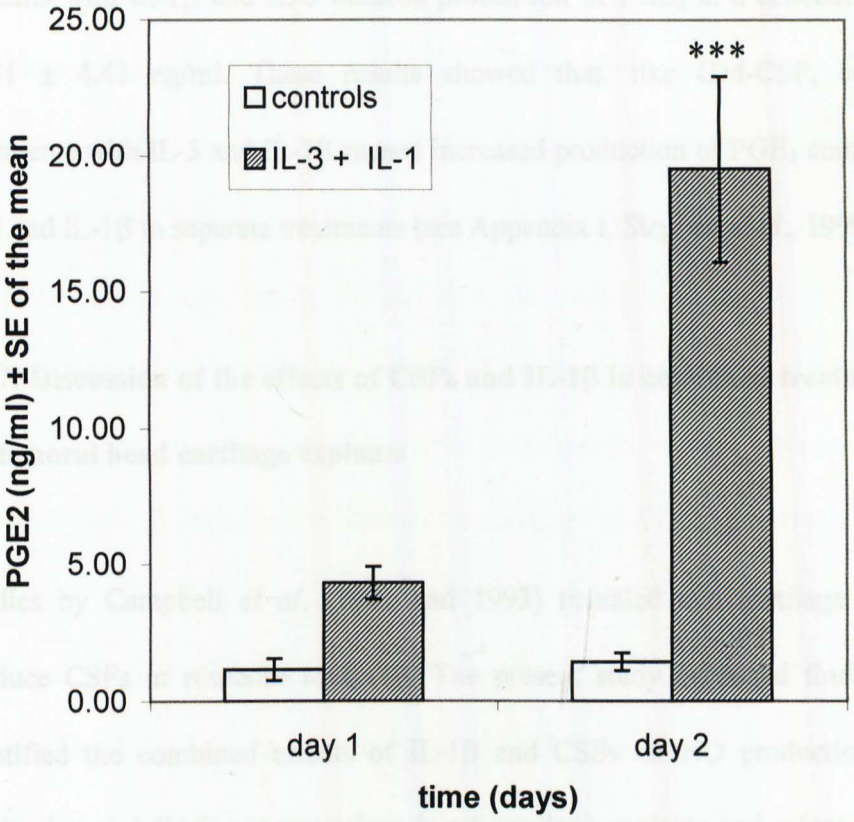


Fig. 5.2.6: Rat femoral head cartilage explants derived from (n=8) were treated with 10ng/ml of IL-3 and 10 ng/ml IL-1β for a 48 hour incubation period with a change of media at 24 hours. PGE_2 concentration was measured in the culture media and compared to controls. Analysis of data using a Students t test showed that IL-3 and IL-1β caused a significant (***$P<0.001$) increase in PGE_2 concentrations in the media on day 2 compared to the same day control group.

Rat cartilage explants treated with IL-3 and IL-1β showed a significant ($p<0.001$) increase in concentration of PGE_2 in the culture media after day 2 compared to the

same day control group. Individual treatments with both IL-1β (Fig 3.1.6) and IL-3 (Fig 4.2.7) induced production of PGE_2 at concentrations of 2.47 ± 0.17 and 10.6 ± 0.61 ng/ml, respectively. However, the combined treatment of cartilage explants with IL-1β and IL-3 induced production of PGE_2 at a concentration of 19.51 ± 4.43 ng/ml. These results showed that, like GM-CSF, combined treatments with IL-3 and IL-1β caused increased production of PGE_2 compared to IL-3 and IL-1β in separate treatments (see Appendix I, Stephan *et al.*, 1999).

5.2.7: Discussion of the effects of CSFs and IL-1β in combined treatments on rat femoral head cartilage explants

Studies by Campbell *et al.* (1991 and 1993) revealed that cartilage explants produce CSFs in response to IL-1β. The present study produced findings that identified the combined effects of IL-1β and CSFs on NO production, PGE_2 production and GAG concentrations in rat cartilage explants and release into the culture media.

GAG concentrations in rat cartilage explants were reduced following treatment with G-CSF and IL-1β for 6 days compared to controls (Fig 5.1.1). This effect was comparable the effect of G-CSF on GAGs in cartilage explants (Fig. 4.1.1). However, in contrast to individual treatments with G-CSF (section 4.1.2) and IL-1β (Fig 3.1.3), increased GAG concentrations were observed in detected in the culture media after 24 hours compared to same day controls following combined

treatment with G-CSF and IL-1β. This demonstrated that the combined treatment of G-CSF and IL-1β was required to cause release of GAGs from rat cartilage explants.

G-CSF and IL-1β also increased production of nitrite in the culture media at 24 intervals during a 6 day time course (Fig. 5.1.3). This compared to individual treatments with G-CSF (section 4.1.4) and IL-1β (Fig. 3.1.5) that did not cause increased nitrite production during a 6 day time course. Elevated nitrite concentrations were detected in individual studies using IL-1β (Fig 3.1.4) and G-CSF (Fig. 4.1.3) where cartilages were in culture for 6 days with a change of media on day 3. Comparison of these results suggest that combined treatment with IL-1β and G-CSF may increase production of nitrite by cartilage explants compared to individual treatments with IL-1β and G-CSF. The combined treatment with G-CSF and IL-1β also increased PGE_2 concentrations in the culture media (Fig. 5.1.4), however, these were comparable to PGE_2 levels produced by treatment with IL-1β (Fig. 3.1.6).

PGE_2 concentrations were increased by GM-CSF and IL-3, when combined with IL-1β. In separate treatments GM-CSF did not cause cartilage explants to produce elevated concentrations of PGE_2 in the culture media (section 4.1.9). However, an increase in PGE_2 production was detected in culture media when combined treatments of GM-CSF and IL-1β were administered to cartilage explants (Fig. 5.1.8).

A similar profile was observed when cartilage explants were treated with IL-3 and IL-1β. Although individual treatment with IL-3 caused elevated PGE_2 production by cartilage explants (Fig. 4.1.3), the combined effect of IL-1β and IL-3 (Fig 5.2.7) was greater than the additive effect of individual treatments with IL-1β and IL-3. These finding demonstrated that both IL-3 and GM-CSF have a synergistic effect with IL-1β with regard to PGE_2 release from rat cartilage explants. This was interesting since the same receptor type and intracellular signalling pathways have been associated with IL-3 and GM-CSF in other cell systems (Mulhern *et al.*, 2000).

The previous experiments in chapters 3, 4 and 5 have shown the responses of rat cartilage explants to CSFs and characterised inflammatory cytokines within an in vitro model. It was planned to develop this model to incorporate a fibroblast cell type and perform co-culture experiments with IL-1β and CSFs. Swiss 3T3 fibroblasts were studied as a potential cell type for developing such a co-culture model. The effects of IL-1β and CSFs on Swiss 3T3 fibroblast monolayers were studied in chapter 6.

Chapter 6:

The effects of IL-1β, TNF-a and CSFs on Swiss 3T3 fibroblasts

6.10: Introduction

In chapters 3, 4 & 5, the effects of IL-1β and CSFs on rat femoral head cartilage explants were studied. The rat explant model provided data with respect to production of PGE_2 and NO, and GAG concentrations in the cartilage explants. Cartilage breakdown has been shown to be mediated by inflammatory mediators in explant models (Seed *et al.,* 1993; Hanglow *et al.*,1995; Stefanovic-*Racic et al.*, 1997; Bird *et. al.*, 1997; Sandy *et al.*, 1999). However, it has also been shown that inflammatory process (Honda *et al.*, 2000) and cartilage breakdown (Seed *et al.* 1993) are both mediated by other cell types. In rheumatoid arthritis fibroblast like cells located in the synovial pannus tissue have been shown to be involved in the pathology of the disease. The present studies have provided findings regarding the activity of cartilage within cartilage explants. It was planned to develop the cartilage explant model by integrating a fibroblast monolayer culture with cartilage explants. These interactions were studied within an in vitro model by modifying the rat cartilage explant model (as used in chapters 3, 4, and 5) to co-culture explants with a monolayer of Swiss 3T3 fibroblasts.

It was desirable to use a fibroblast cell line that did not release GAGs into the culture media. This criteria was crucial to clarify the GAG concentrations detected in the culture media were derived from cartilage explants and not released by fibroblasts. It was also desirable to use a fibroblast cell line that was responsive to IL-1β. Therefore, prior to comencing co-culture experiments a preliminary study was performed using fibroblast monolayers to determine if IL-1β and CSFs influenced their activity with respect to release into the media of GAGs, nitrite and

PGE_2. It was anticipated that Swiss 3T3 fibroblasts would be responsive to IL1β since studies have shown that Swiss 3T3 fibroblasts produce NO and PGE_2 in response to IL-1β (Burch *et al.*, 1989). The use of fibroblasts derived from the synovium was considered, however, the availability of this cell type prohibited its use in this study.

6.1.1: Swiss 3T3 fibroblasts and GAGs

A study by Tsiganos *et al.*, (1982) showed that 3T3 cells possessed GAG-like moeities on the surface of their extracellular matrix. However, it was not clear if these molecules would be released into the culture media and be detectable using the DMB GAG binding assay as described by Goldberg and Kolibas, (1990). This was explored using cultured fibroblasts monolayers in 24 well plates with a control group and individual treatment groups consisting of (n=4) IL-1β, G-CSF, GM-CSF, M-CSF and IL-3 at a concentration of 10 ng/ml. Monolayers were cultured from 80 % confluence for 6 days periods with a change of media at 24 hour intervals. Media samples were collected and GAG concentrations were quantified. GAGs were not detected control groups media or in media from fibroblasts treated with IL-1β and CSFs.

The reponse of Swiss 3T3 fibroblasts to IL-1β were also measured during a 6 day period at 24 hour intervals. Nitrite concentrations were measured throughout the 6 day period and PGE_2 concentrations were measured following days 1 and 2. The results from these studies are illustrated in Figs. 6.1.2 and 6.1.3.

6.1.2 Effects of IL-1β on nitrite production by Swiss 3T3 cells

Fig 6.1.2: Effect of IL-1β on nitrite production by Swiss 3T3 fibroblasts during a 6 day time course.

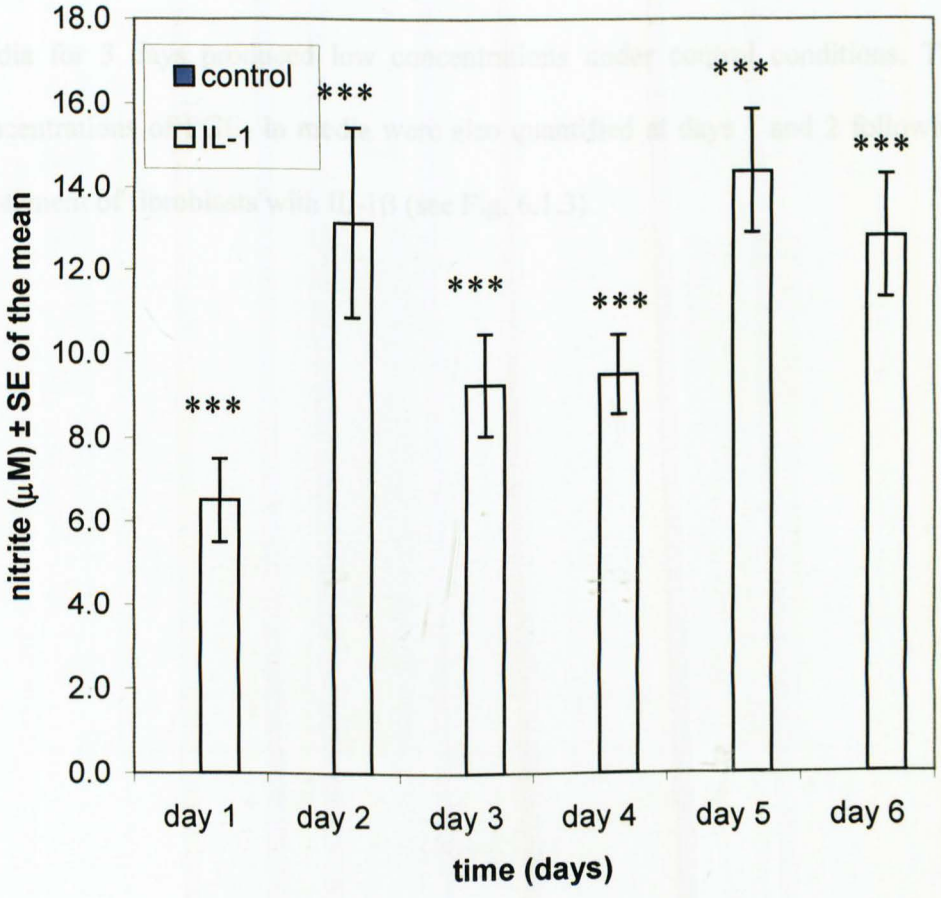


Fig. 6.1.2: A time course of nitrite production by Swiss 3T3 fibroblasts (n=4) in tissue culture supernatant was measured during a six day period at 24 hour intervals following treatment with 10 ng/ml of IL-1β. Nitrite was not detected in control groups. Analysis of data using a Students t test showed that IL-1β caused a significant (***P<0.001) increase in nitrite concentrations in the media at all measured time points compared respective control groups.

The measurement of nitrite in culture media at 24 intervals from fibroblast monolayers revealed that nitrite was not produced in control groups. Conversely, treatment with IL-1β increased caused nitrite to be detected at all time points in the six day culture. Supplemental experiments showed that fibroblasts cultured in media for 3 days produced low concentrations under control conditions. The concentrations of PGE_2 in media were also quantified at days 1 and 2 following treatement of fibroblasts with IL-1β (see Fig. 6.1.3).

6.1.3: Effect of IL-1β on PGE_2 production by Swiss 3T3 fibroblasts

Fig 6.1.3: Effect of IL-1β on PGE2 production by Swiss 3T3 fibroblasts.

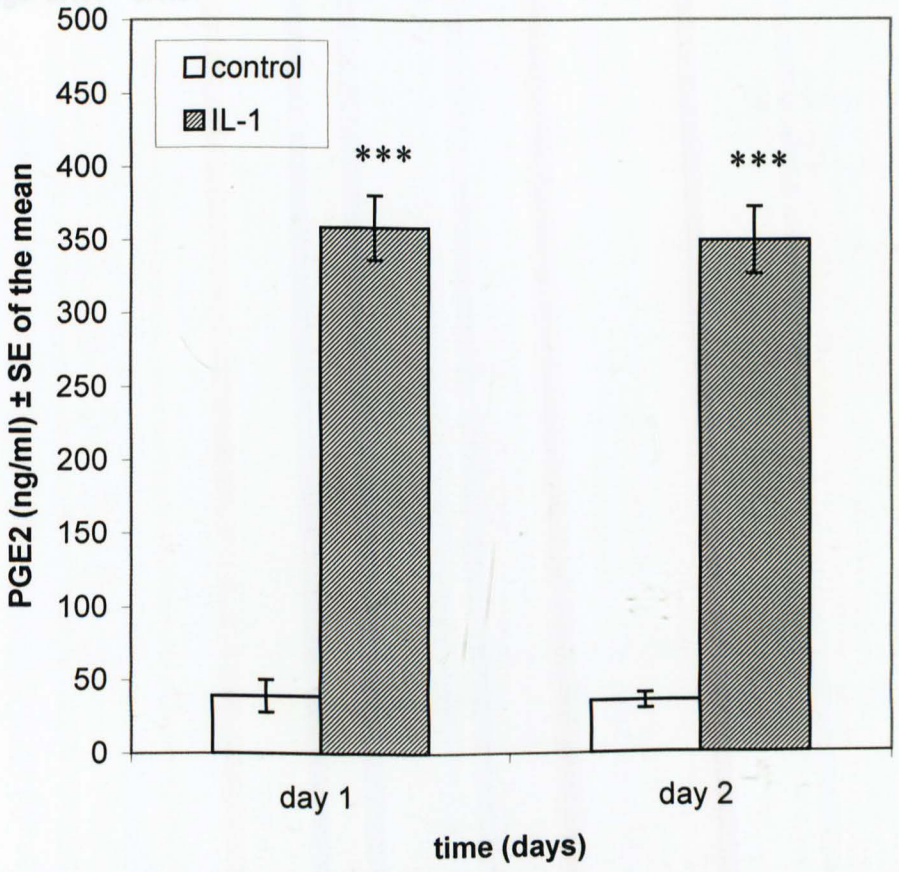


Fig. 6.1.3: Swiss 3T3 fibroblasts (n=4) were treated with 10ng/ml of IL-1β for a 48 hour incubation period with a change of media at 24 hours. PGE_2 concentrations were measured in the culture media and compared to the control group. Analysis of data using a Students t test showed that IL-1β caused a significant (***$P<0.001$) increase in PGE_2 concentrations in the media on days 1 and 2 compared to the same day control group.

PGE_2 concentrations in the culture media were significantly ($p<0.001$) increased on both days 1 and 2 following treatment with IL-1β. Fibroblast monolayers were

also treated with TNF-α to determine its effects on nitrite and PGE_2 concentrations. A dose of 100 ng/ml of TNFα was selected to allow comparison of results obtained in cartilage explant experiments. The results are illustrated in Figs. 6.1.4 - 6.1.5.

6.1.4: Effect of TNFα on nitrite production by Swiss 3T3 fibroblasts

Fig 6.1.4: Effect of TNF-α on nitrite production by Swiss 3T3 fibroblasts during a 6 day time course

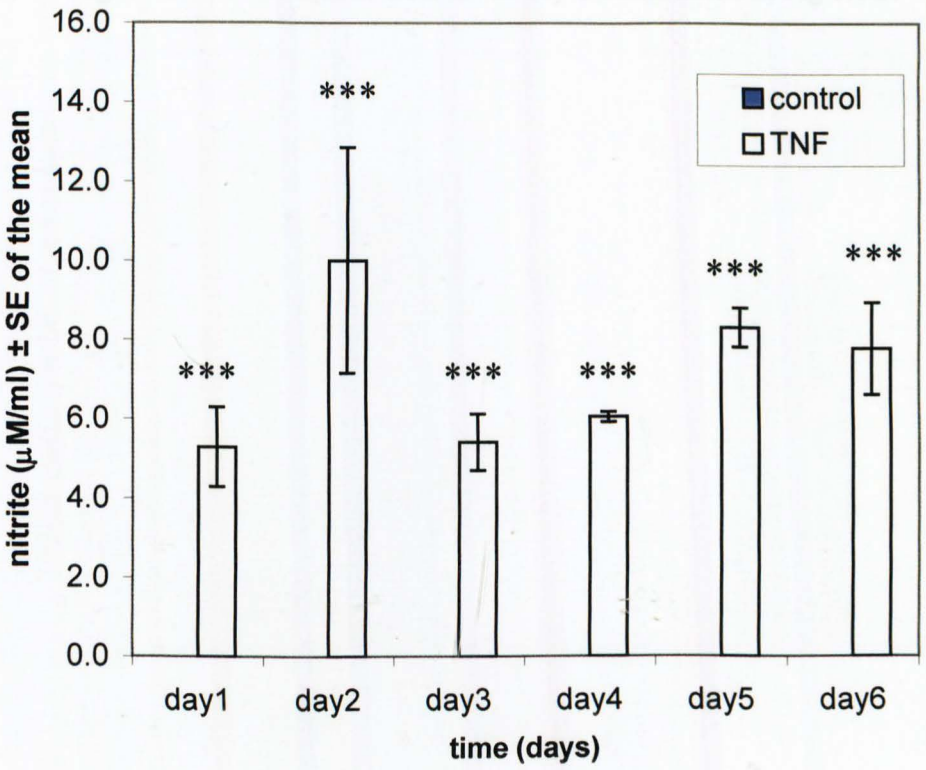


Fig. 6.1.4: A time course of nitrite production by Swiss 3T3 fibroblasts (n=4) in tissue culture supernatant was measured during a six day period at 24 hour intervals following stimulation with 100 ng/ml of TNFα. Nitrite was not detected in control groups. Analysis of data using a Students t test showed that TNFα caused a significant (***P<0.001) increase in nitrite concentrations in the media at all measured time points compared with respective control groups.

The measurement of nitrite in fibroblast monolayers revealed that nitrite production was not produced in control groups. Treatment with TNF-α resulted in an increase of media nitrite levels at all time points in the six day culture.

However, concentrations of nitrite produced were lower than concentrations detected following treatment with IL-1β (Fig 6.1.2). The concentrations of PGE_2 in media were also quantified at days 1 and 2 following treatement of fibroblasts with 100ng/nl TNFα. The results from this study are illustrated in Fig 6.1.5.

6.1.5: Effect of TNF-α on PGE_2 production by Swiss 3T3 fibroblasts

Fig 6.1.5: Effect of TNF-α on PGE2 production by Swiss 3T3 fibroblasts.

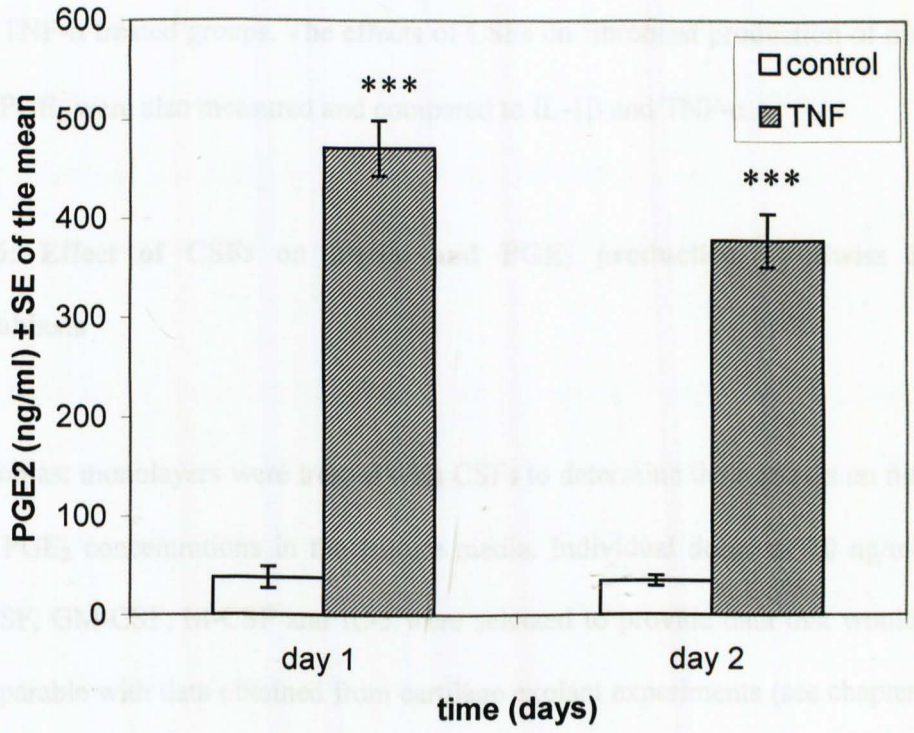


Fig. 6.1.5: Swiss 3T3 fibroblasts (n=4) were treated with 100ng/ml of TNFα for a 48 hour incubation period with a change of media at 24 hours. PGE_2 concentrations were measured in the culture media and compared to the control group. Analysis of data using a Students t test showed that TNFα caused a significant (***P<0.001) increase in PGE_2 concentrations in the media on days 1 and 2 compared to the same day control group.

PGE_2 concentrations in the culture media were significantly ($p<0.001$) increased on both days 1 and 2 following treatment with TNF-α. These experiments showed that both IL-1β and TNF-α caused Swiss 3T3 fibroblasts to produce elevated

concentrations of nitrite and PGE_2 in vitro compared to controls. Cell viability was also assessed after 48 hours of treatments with IL1β and TNFα, with a change of media at 24 hours to determine if IL-1β and TNFα increased cell death in fibroblast monolayers. Cell viabilities were found to be >95 % in controls, IL-1β and TNF-α treated groups. The effects of CSFs on fibroblast production of nitrite and PGE_2 were also measured and compared to IL-1β and TNF-α.

6.1.6: Effect of CSFs on nitrite and PGE_2 production by Swiss 3T3 fibroblasts

Fibroblast monolayers were treated with CSFs to determine their effects on nitrite and PGE_2 concentrations in the culture media. Individual doses of 10 ng/ml of G-CSF, GM-CSF, M-CSF and IL-3 were selected to provide data that would be comparable with data obtained from cartilage explant experiments (see chapter 4). Fibroblasts were treated with CSFs during a 6 day culture period and media was changed at 24 hour intervals. Nitrite was not detected in media collected from any of the CSF treated groups of fibroblast monolayers (n=4) at any time point during the 6 day culture period. Basal PGE_2 concentrations were produced by both control and CSF treated fibroblasts, as in controls in experiments using IL-1β and TNFα. However, the CSFs used in these experiments did not significantly change concentrations of PGE_2 detected in the media compared to respective controls. Cell viability was also assessed to determine if CSFs increased cell death in

fibroblast monolayers. Cell viabilities were found to be > 95 % in controls and CSF treated groups.

It was observed that the combined treatment of selected CSFs with IL-1β caused increased responsiveness of cartilage explants with respect to PGE_2 and nitrite concentrations produced. G-CSF combined with IL-1β caused increased nitrite production and combined treatments with GM-CSF / IL-1β and IL-3 / IL-1β caused increased production of PGE_2 by rat cartilage explants.

The response of fibroblast monolayers was determined using similar combined treatment regime with CSFs and IL-1β. Nitrite was measured during a 6 day culture period with a change of media at 24 hour intervals. PGE_2 was quantified at days 1 and 2 of culture with a change of media at 24 hours. The results from this study are illustrated in sections 6.1.7-6.1.8.

6.1.7: Effect of IL-1β and CSFs on nitrite production by Swiss 3T3 fibroblasts

Fibroblast monolayers were treated with IL-1β and CSFs to determine their effects on nitrite concentrations in the culture media. A dose of 10 ng/ml IL-1β was combined with individual doses of 10 ng/ml of G-CSF, GM-CSF, M-CSF and IL-3. This provided data that was comparable to results obtained in cartilage explant experiments (see chapter 5) and results obtained using IL-1β (Figs. 6.1.2

and 6.1.3) and individual treatments of fibroblast monolayers with CSFs (section 6.1.6). The results of combined treatments are illustrated in Fig. 6.1.7.

Fig 6.1.7: Effects of CSFs combined with IL-1β on nitrite production by Swiss 3T3 fibroblasts

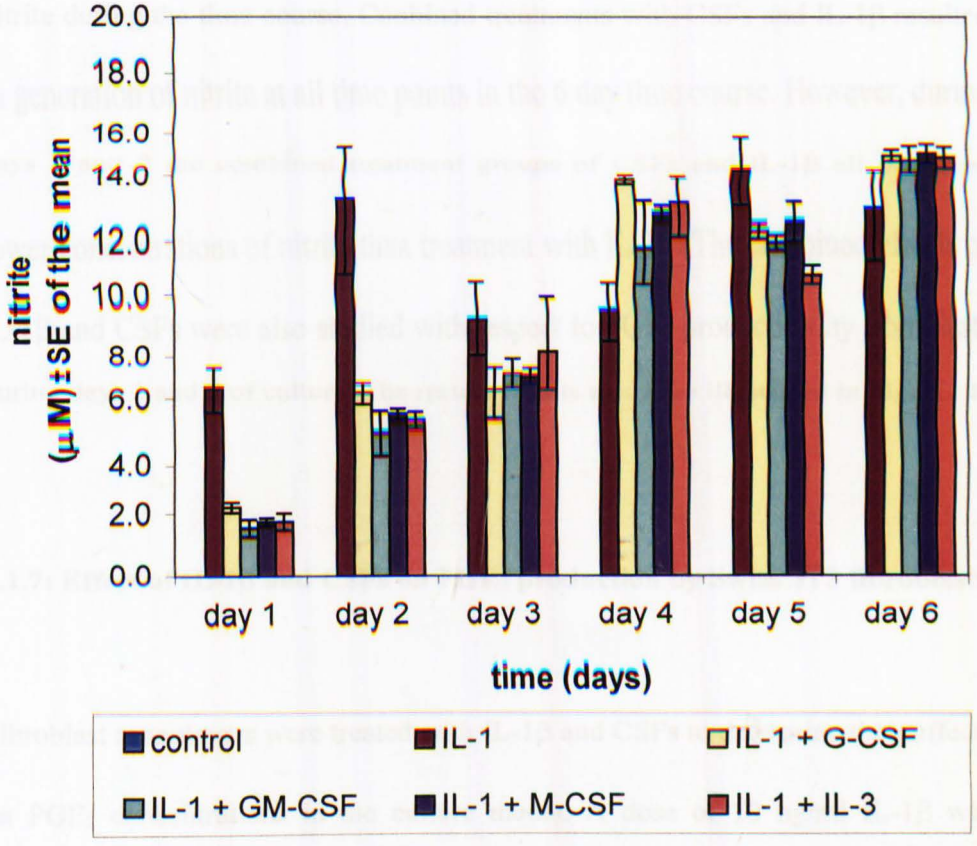


Fig. 6.1.7: A time course of nitrite production by Swiss 3T3 fibroblasts (n=4) in tissue culture supernatant was measured during a six day period at 24 hour intervals following treatment with 10 ng/ml of IL-1β combined with 10 ng/ml doses of CSFs. Nitrite was not detected in control groups. Nitrite production was observed in the IL-1β treated monolayers at all time points. Nitrite production in combined groups increased from day 1-6 with highest levels of nitrite detected at the 6 day period in all combined treatment groups. During days 1 and 2 the

concentration of nitrite in all combined IL-1β and CSF treatment groups was lower than that in the IL-1β treated group

IL-1β caused fibroblasts to generate nitrite in the culture media at all time points in a 6 day time course. Control fibroblasts did not produce detectable levels of nitrite during the time course. Conbined treatments with CSFs and IL-1β resulted in generation of nitrite at all time points in the 6 day time course. However, during days 1 and 2 the combined treatment groups of CSFs and IL-1β all produced lower concentrations of nitrite than treatment with IL-1β. The combined effects of IL-1β and CSFs were also studied with respect to PGE_2 production by fibroblasts during days 1 and 2 of culture. The results of this study are illustrated in Fig. 6.1.8.

6.1.7: Effect of IL-1β and CSFs on PGE_2 production by Swiss 3T3 fibroblasts

Fibroblast monolayers were treated with IL-1β and CSFs to determine their effects on PGE_2 concentrations in the culture media. A dose of 10 ng/ml IL-1β was combined with individual doses of 10 ng/ml of G-CSF, GM-CSF, M-CSF and IL-3. These doses were selected to provide comparable data to results obtained in cartilage explant experiments (see chapter 5) and results obtained using IL-1β (Fig 6.1.3) and individual treatments of fibroblast monolayers with CSFs (section 6.1.6). The results of combined treatments are illustrated in Fig. 6.1.8.

Fig. 6.1.8: Effect of CSFs combined with IL-1β on PGE2 production by Swiss 3T3 fibroblasts

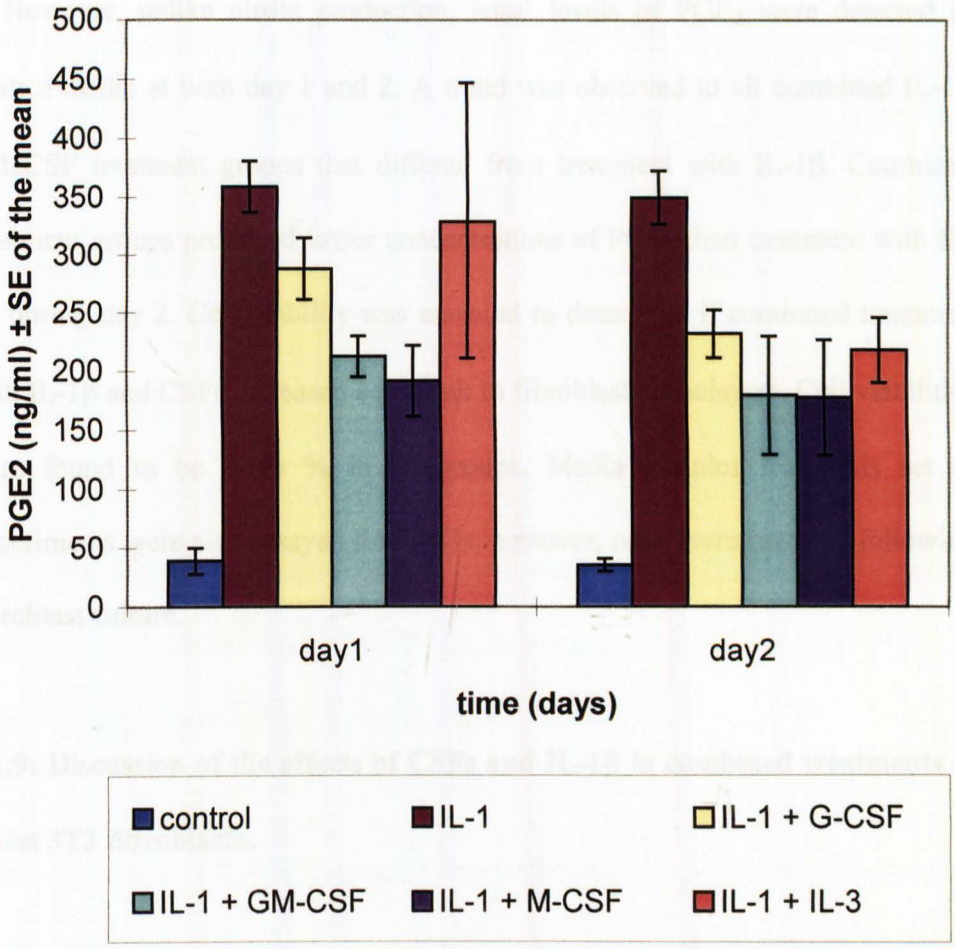


Fig. 6.1.8: Swiss 3T3 fibroblasts (n=4) were treated with 10ng/ml of IL-1β and 10 ng/ml of CSFs for a 48 hour incubation period with a change of media at 24 hours. PGE_2 concentrations were measured in the culture media and compared to controls. Control fibroblasts produced basal levels of PGE_2 after days 1 and 2. Fibroblasts with IL-1β caused an increase in PGE_2 concentrations compared to the control group on days 1 and 2. Combined treatments of IL-1β and CSFs also produced an increase in PGE_2 concentrations compared to the control group.

During day 2 the concentrations of PGE_2 in all combined IL-1β and CSF treatment groups was lower than that in the IL-1β treated group.

IL-1β caused fibroblasts to generate PGE_2 in the culture media at both day 1 and 2. However, unlike nitrite production, basal levels of PGE_2 were detected in control media at both day 1 and 2. A trend was observed in all combined IL-1β and CSF treatment groups that differed from treatment with IL-1β. Combined treatment groups produced lower concentrations of PGE_2 than treatment with IL-1β during day 2. Cell viability was assessed to determine if combined treatment with IL-1β and CSFs increased cell death in fibroblast monolayers. Cell viabilities were found to be >95 % in all groups. Media samples from this set of experiments were also assayed for GAGs, however, none were detected following fibroblast culture.

6.1.9: Discussion of the effects of CSFs and IL-1β in combined treatments of Swiss 3T3 fibroblasts

The primary objectives of this study were to deterimine if Swiss 3T3 fibroblast release GAGs into the culture media and to determine if Swiss 3T3 fibroblasts were responsive to IL-1β and CSFs. Despite the report by Tsiganos *et al.*, (1982) that indicated 3T3 cell possess GAG-like moeities on the surface of their extracellular membrane, GAGs were not detected in the culture media in control or cytokine treatment conditions (section 6.1.1). This result indicated that Swiss 3T3 fibroblasts were suitable for co-culture with rat cartilage explants since GAGs

detected in the media would be derived from cartilage explants. It was assumed that the presence of rat cartilage explants in the co-culture environment would not have caused release of GAGs by fibroblast monolayers.

Fibroblast monolayers were also studied to determine their responsiveness to IL-1β and TNFα with respect to NO and PGE_2 production. Burch *et al.*, (1989) showed that fibroblasts produce PGE_2 and show elevated expression of COX enzymes in response to IL-1β. Therefore, it was anticipated that IL-1β would caused increased production of nitrite and PGE_2 in the culture media. Increased concentrations of nitrite (Fig 6.1.2) and PGE_2 (Fig 6.1.3) were indeed detected in culture media following treatment with IL-1β. PGE_2 levels produced by control fibroblasts and IL-1β treated fibroblasts were approximately 30 and 350 ng/ml, respectively, compared to control and IL-1β treated cartilage explants (Fig. 3.2.1) approximately 1 and 2.5 ng/ml, respectively.

It was also shown that TNFα produced a similar increase in levels of nitrite (Fig. 6.1.3) and PGE_2 (Fig. 6.1.4) production by fibroblast monolayers. PGE_2 levels produced by control fibroblasts and TNF-α treated fibroblasts (approximately 30 and 400 ng/ml, respectively) were compared to control and TNF-α treated cartilage explants (Fig 3.1.6, approximately 1 and 2 ng/ml, respectively). It was concluded from these studies that Swiss 3T3 fibroblasts are responsive to IL-1β and TNFα treatment with regard to nitrite and PGE_2 production.

These results fulfiled the criteria for use of Swiss 3T3 fibroblasts in a co-culture system with rat femoral head cartilages. It was also concluded that Swiss 3T3 fibroblasts monolayers produce elevated concentrations of PGE_2 in control conditions and following treatment with IL-1β compared to comparative concentrations produced by rat cartilage explants in similar conditions.

Treatment of fibroblast monolayers with CSFs did not cause a significant change in nitrite, PGE_2 or GAGs detected in the media samples. CSFs were also combined with IL-1β as in previous studies with cartilage explants (chapter 5). It was observed that nitrite concentrations were elevated compared to controls following all combined treatment IL-1β and CSF. However, it was noted that nitrite levels induced by combined treatments were less than nitrite levels induced by IL-1β on days 1 and 2 (Fig. 6.1.7). A similar effect was observed when measuring concentrations of PGE_2. The combined treatments with IL-1β and CSFs induced PGE_2 concentrations that were less than those induced by IL-1β on day 2 (Fig. 6.1.8). It was concluded that these observations resulted from a non-specific effect of combined treatments since it was observed in all combination treatments.

This series of experiments confirmed the suitability of using Swiss 3T3 fibroblast activity following treatment with IL-1β, TNF-α and CSFs. Swiss 3T3 fibroblasts were therefore used in fibroblast-cartilage co-culture experiments, as described in chapter 7.

Chapter 7:

Effects of IL-1β and CSFs on cartilage breakdown in a co-culture model using rat femoral head cartilages and Swiss 3T3 fibroblasts.

7.10 Introduction

IL-1β was shown to increase in PGE_2 and NO production by rat femoral head explants in a previous study (see chapter 3). However, IL-1β did not cause loss of GAGs into the media from the rat cartilage. This contradicted other studies (Seed et al., 1993; Hanglow et al.,1995; Stefanovic-Racic et al. 1997; Bird et. al. 1997; Sandy et al., 1999; Spirito et al., 1995; Bottomley et. al., 1997) that showed IL-1β caused GAG loss from cartilage explants.

The cartilage explants was therefore integrated with a fibroblasts monolayer to produce a fibroblast-cartilage co-culture model. Murine Swiss 3T3 fibroblasts were selected for this study since they did not release GAGs into the culture media and were responsive to IL-1β treatment with regard to nitrite and PGE2 production (see chapter 6). The aim of this study was to determine if the presence of fibroblasts in the model would cause an increase in loss of GAGs from the cartilage matrix under control and cytokine treatment conditions. IL-1β was used as a treatment co-culture system to determine if GAG loss was induced in rat cartilage explants. Since GAGs were not produced in the media of fibroblasts alone, loss of GAGs were assumed to be a result of cartilage breakdown in the co-culture system. Both nitrite and PGE_2 were also measured in the co-culture media and compared to concentrations of GAGs detected in the culture media during the experiments. GAG release and nitrite production were measured during a six day

time course to compare with experiments performed using cartilage explants (chapters 3, 4 and 5) and fibroblasts (chapter 6). PGE_2 concentrations were measured at days 1 and 2 to compare with experiments performed using cartilage explants (chapters 3, 4 and 5) and fibroblasts (chapter 6). Fibroblast viabilities were also assessed following 6 day co-culture experiments to determine if cell death occured in fibroblast monolayers. The results of IL-1β treatment in the co-culture system are illustrated in Figs. 7.1.1-7.1.3.

7.1.1: Effect of IL-1β on GAG release from cartilage explants co-cultured with Swiss 3T3 fibroblasts

Fig. 7.1.1: Effect of IL-1β on GAG release into culture media from rat femoral head cartilage co-cultured with Swiss 3T3 Fibroblasts during a six day time course.

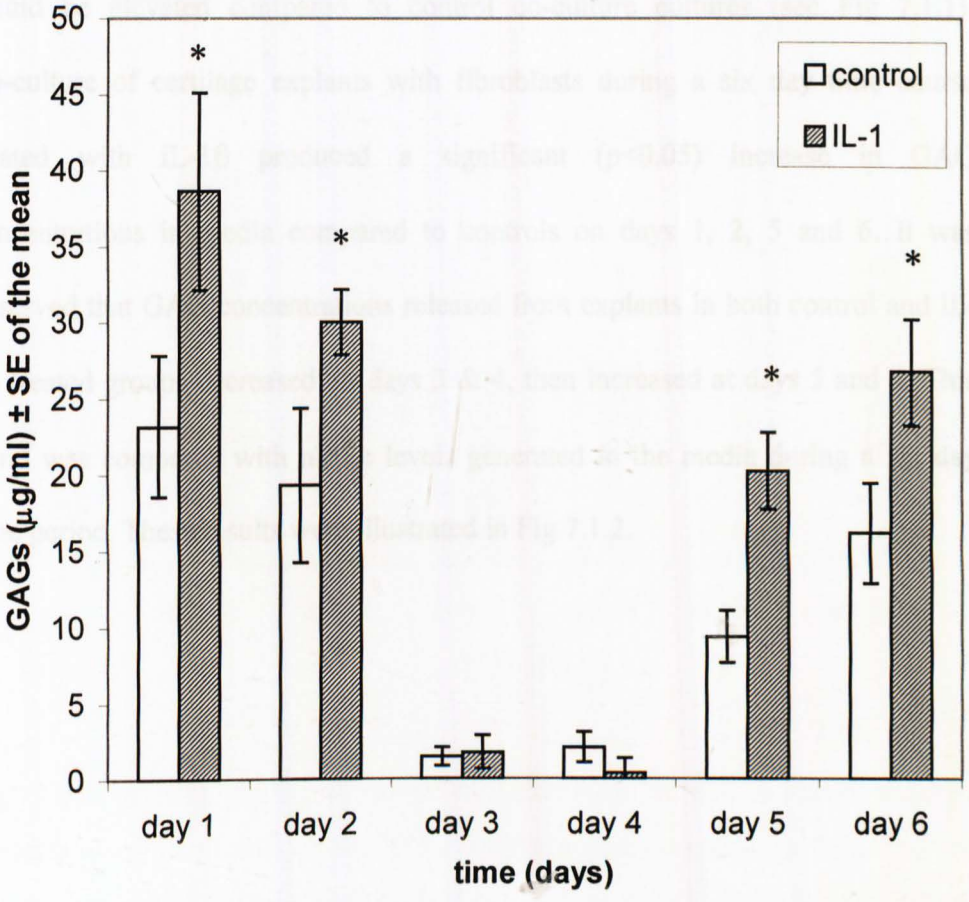


Fig. 7.1.1: A time course of GAG release by rat femoral head cartilage explants co-cultured with fibroblasts (n=6) was measured in tissue culture supernatants during a six day period at 24 hour intervals following treatment with 10 ng/ml of IL-1β. Analysis of data using a Students t test showed that IL-1β produced a significant (*= $P<0.05$) increase GAG concentrations in the culture media compared to the control group during days 1, 2, 4 and 5.

GAG concentrations in culture media from co-culture controls showed a trend of being elevated compared to GAG concentrations in control groups within rat cartilage explant experiments (Fig 3.1.3). This demonstrated that the presence of fibroblasts caused increased loss of GAGs from cartilage explants. The co-cultures were treated with IL-1β at a concentration of 10ng/ml to determine if GAG loss would be elevated compared to control co-culture cultures (see Fig 7.1.1). Co-culture of cartilage explants with fibroblasts during a six day time course treated with IL-1β produced a significant ($p<0.05$) increase in GAG concentrations in media compared to controls on days 1, 2, 5 and 6. It was observed that GAG concentrations released from explants in both control and IL-1β treated groups decreased on days 3 & 4, then increased at days 5 and 6. This trend was compared with nitrite levels generated in the media during a six day time period. These results were illustrated in Fig 7.1.2.

7.1.2: Effect of IL-1β on nitrite produced by a co-culture of rat cartilage explants with Swiss 3T3 fibroblasts

Fig. 7.1.2: Effect of IL-1β on nitrite production by a co-culture of rat cartilage explants with Swiss 3T3 fibroblasts

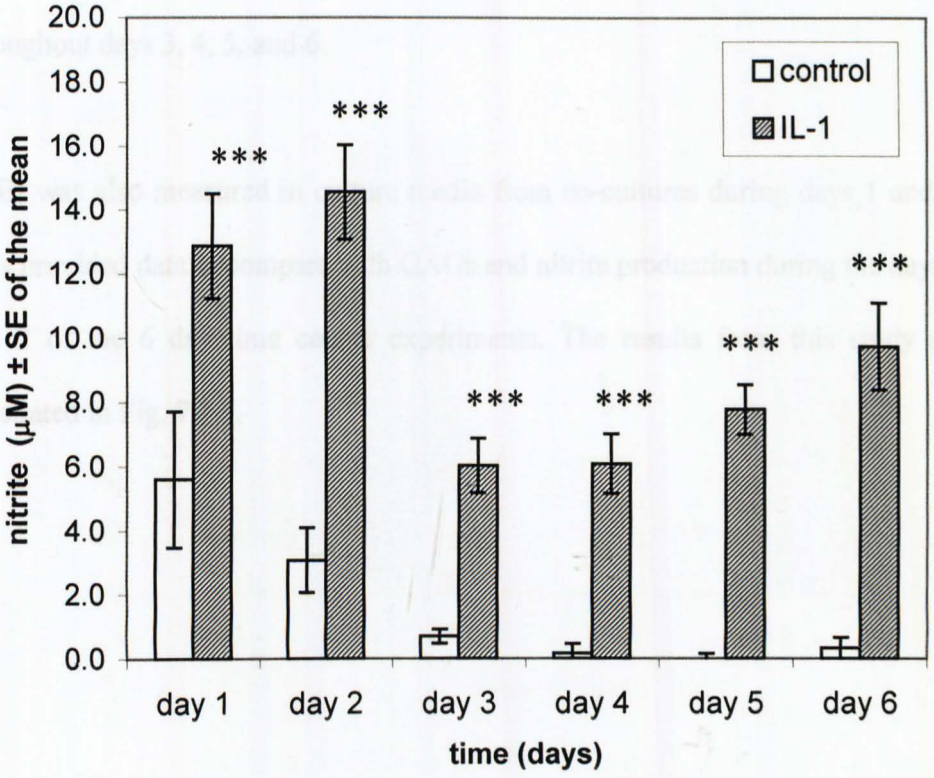


Fig. 7.1.2: A time course of nitrite production by rat femoral head cartilage explants co-cultured with Swiss 3T3 fibroblasts was measured in tissue culture supernatant during a six day period at 24 hour intervals following stimulation with 10 ng/ml of IL-1β. Analysis of data using a Students t test showed that nitrite concentrations in the culture media were significantly ($p<0.001$) higher in the IL-1β treated group compared to the same time control group at all time points.

Co-culture of cartilage explants with fibroblasts during a six day time course produced a significant (***$P<0.001$) increase in nitrite concentrations in media

compared to controls at all time points. It was observed that nitrite concentrations decreased at days 3 and 4 in IL-1β treated groups then increased on days 5 and 6. This resembled the trend observed in the GAG loss profile from the IL-1β treated co-cultures during the 6 day time course. Conversely, nitrite concentrations in media from the control group decreased from days 1 and 2 and remained low throughout days 3, 4, 5, and 6.

PGE_2 was also measured in culture media from co-cultures during days 1 and 2. This provided data to compare with GAGs and nitrite production during the days 1 and 2 of the 6 day time course experiments. The results from this study are illustrated in Fig. 7.1.3.

7.13: Effect of IL-1β on concentration of PGE_2 produced by a co-culture of rat cartilage explants with Swiss 3T3 fibroblasts

Fig. 7.1.3: Effect of IL-1β on PGE2 production by a co-culture of rat cartilage explants with Swiss 3T3 fibroblasts

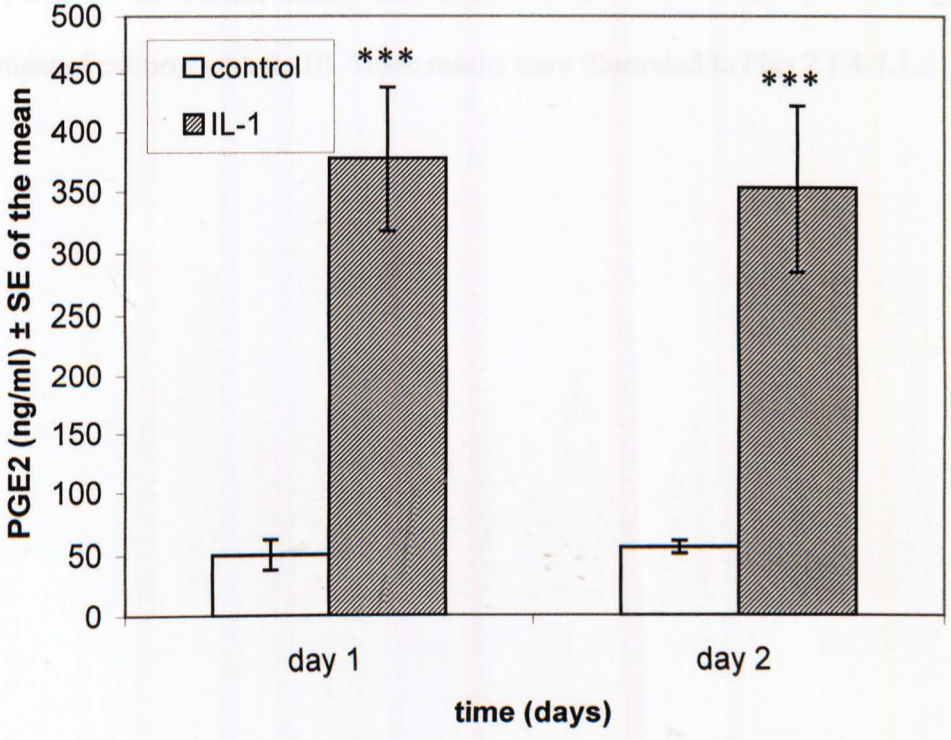


Fig. 7.1.3: Rat femoral head cartilage explants (n=8) co-cultured with Swiss 3T3 fibroblasts were treated with 10ng/ml of IL-1β for a 2 day incubation period with a change of media at 24 hours. PGE_2 concentrations were measured in the culture media and compared to the control group. Analysis of data using a Students t test showed that concentrations of PGE_2 in the culture media from the IL-1β treated group were significantly ($p<0.001$) elevated compared to same time controls at days 1 and 2.

IL-1β treatment resulted in a significant (***$P<0.001$) increase of PGE_2 production after days 1 and 2. The concentration of PGE_2 detected in the

co-culture system following treatment with IL-1β (Fig. 7.1.3) was comparable to the sum of the concentrations of PGE_2 produced by cartilages (Fig. 3.1.6) and fibroblasts (Fig. 6.1.3). The effects of the CSFs on Swiss 3T3 fibroblasts co-cultured with cartilage explants were also studied with respect to GAGs, nitrite and PGE_2 in the culture media and compared to results obtained following treatment of cultures with IL-1β. These results were illustrated in Figs 7.1.4-7.1.6.

7.1.4: Effects of CSFs on GAG release from cartilage explants co-cultured with Swiss 3T3 fibroblasts

Fig. 7.1.4: Effect of CSFs on GAG release into culture media from rat femoral head cartilage co-cultured with Swiss 3T3 Fibroblasts during a six day time course.

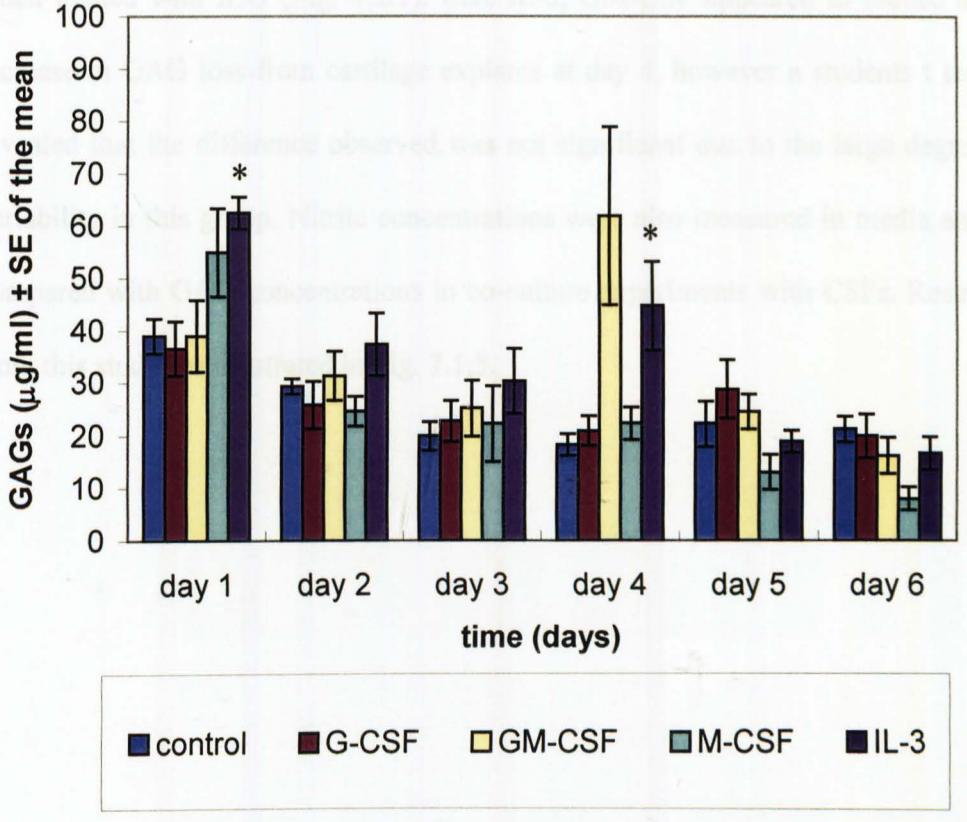


Fig. 7.1.4: A time course of GAG release by rat femoral head cartilage explants co-cultured with fibroblasts (n=6) was measured in tissue culture supernatants during a six day period at 24 hour intervals following individuall treatments with 10 ng/ml of G-CSF, GM-CSF, M-CSF and IL-3. Analysis of data using a Students t test showed that concentrations of GAGs in the culture media from the IL-3 treated group were significantly ($p<0.05$) elevated compared to same time controls on days 1 and 4.

IL-3 produce a significant ($p<0.05$) increase in GAG release from cartilage explants on days 1 and 4. This differed from treatment with IL-3 that did not increase GAG release from cartilage explants (section 4.2.4). However, in the previous study cartilage explants produced increased levels of PGE_2 in media when treated with IL-3 (Fig. 4.2.7). Like IL-3, GM-CSF appeared to induce an increase in GAG loss from cartilage explants at day 4, however a students t test revealed that the difference observed was not significant due to the large degree variability in this group. Nitrite concentrations were also measured in media and compared with GAG concentrations in co-culture experiments with CSFs. Result from this study are illustrated in Fig. 7.1.5.

7.1.5: Effect of CSFs on nitrite produced by a co-culture of rat cartilage explants with Swiss 3T3 fibroblasts

Fig. 7.1.5: Effect of CSFs on nitrite production by a co-culture of rat cartilage explants with Swiss 3T3 fibroblasts

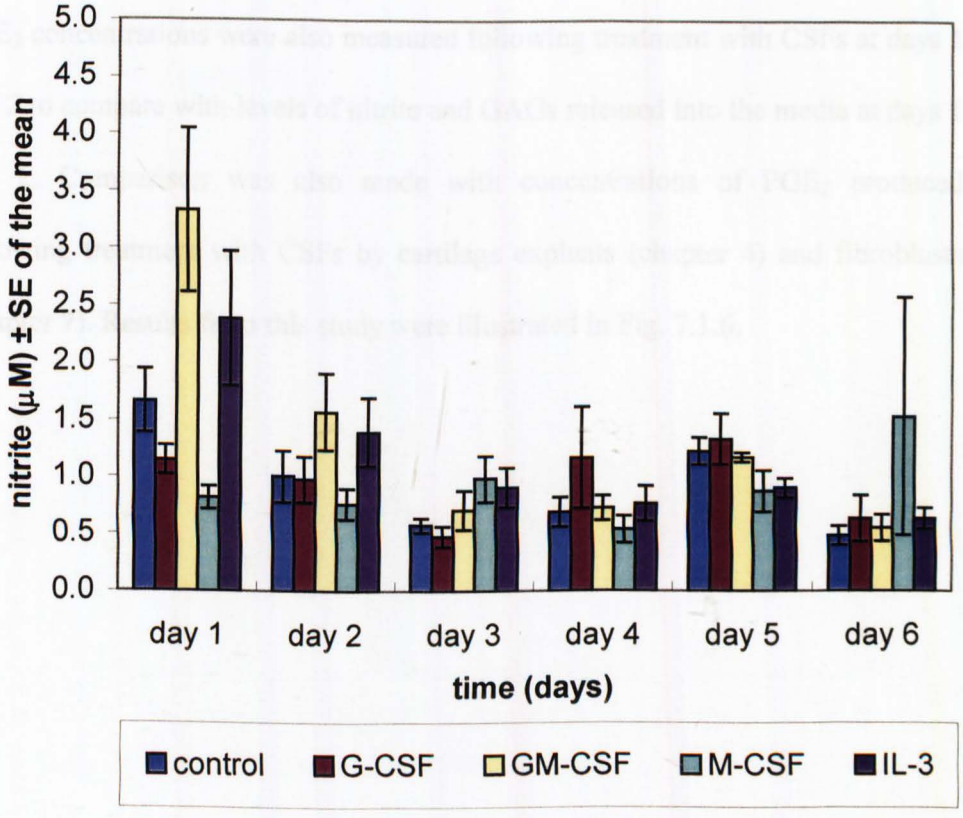


Fig. 7.1.5: A time course of nitrite production by rat femoral head cartilage explants co-cultured with Swiss 3T3 fibroblasts was measured in tissue culture supernatant was measured during a six day period at 24 hour intervals following stimulation with 10 ng/ml of G-CSF, GM-CSF, M-CSF and IL-3. Analysis of data using a Students t test showed that concentrations of nitrite in the culture media were not changed by treatment with CSFs compared to the control group.

Treatment with CSFs did not cause significant changes of nitrite concentrations in media compared to controls at 24 hour intervals during a 6 day time course. This compared with CSF treatments in cartilage experiments (chapter 4) and in fibroblast experiments (chapter 6) in which no significant differences were observed from controls at 24 hour intervals during a 6 day time course.

PGE_2 concentrations were also measured following treatment with CSFs at days 1 and 2 to compare with levels of nitrite and GAGs released into the media at days 1 and 2. Comparison was also made with concentrations of PGE_2 produced following treatment with CSFs by cartilage explants (chapter 4) and fibroblasts (chapter 7). Results from this study were illustrated in Fig. 7.1.6.

7.1.6: Effect of CSFs on concentration of PGE_2 produced by a co-culture of rat cartilage explants with Swiss 3T3 fibroblasts

Fig. 7.1.6: Effect of CSFs on PGE2 production by a co-culture of rat cartilage explants with Swiss 3T3 fibroblasts

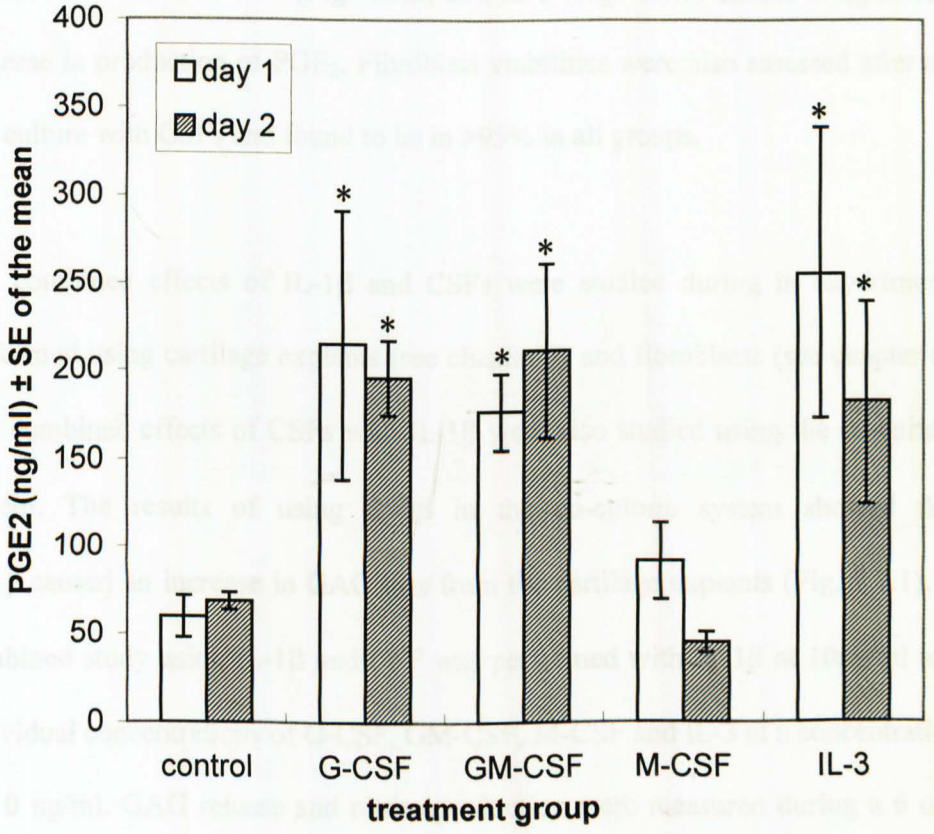


Fig. 7.1.6: Rat femoral head cartilage explants (n=8) co-cultured with Swiss 3T3 fibroblasts were treated with individual doses of 10ng/ml of G-CSF, GM-CSF, M-CSF and IL-3 for a 2 day incubation with a change of media at 24 hours. PGE_2 concentrations were measured in the culture media and compared to the control group. Analysis of data using a Students t test showed that concentrations of PGE_2 in the culture media were significantly ($p<0.05$) increased by G-CSF, GM-CSF and IL-3 on days 1 and 2.

G-CSF, GM-CSF and IL-3 all caused a significant increase ($P<0.05$) in PGE_2 production in the co-culture system compared to controls. This compared to previous experiments with fibroblasts where G-CSF, GM-CSF, M-CSF and IL-3 did not have an effect on PGE_2 production (section 6.1.6) and previous cartilage experiments where G-CSF (Fig. 4.1.5) and IL-3 (Fig. 4.2.7) caused a significant increase in production of PGE_2. Fibroblast viabilities were also assessed after a 6 day culture with CSFs and found to be in >95% in all groups.

The combined effects of IL-1β and CSFs were studied during in experiments performed using cartilage explants (see chapter 5) and fibroblasts (see chapter 6). The combined effects of CSFs with IL-1β were also studied using the co-culture system. The results of using IL-1β in the co-culture system showed that IL-1β caused an increase in GAG loss from the cartilage explants (Fig. 7.1.1). A combined study using IL-1β and CSF was performed with IL-1β at 10ng/ml and individual concentrations of G-CSF, GM-CSF, M-CSF and IL-3 at a concentration of 10 ng/ml. GAG release and nitrite production were measured during a 6 day time course and PGE_2 concentrations were measured at days 1 and 2. Fibroblast viabilities were also assessed following co-culture experiments. The results from these studies were illustrated in Figs 7.1.7-7.1.9.

7.1.7: Effects of IL-1β and CSFs on GAG release from cartilage explants co-cultured with Swiss 3T3 fibroblasts

Fig. 7.1.7: Effect of IL-1β and CSFs on GAG release into culture media from rat femoral head cartilage co-cultured with Swiss 3T3 fibroblasts during a six day time course.

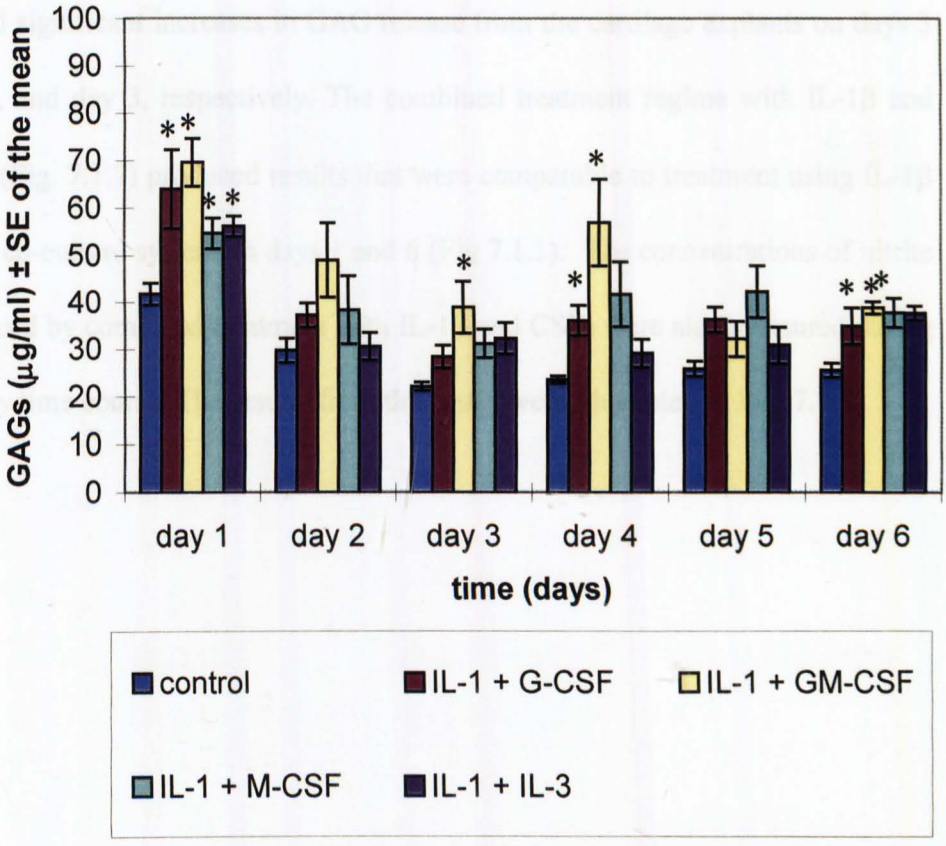


Fig. 7.17: A time course of GAG release by rat femoral head cartilage explants co-cultured with fibroblasts (n=6) was measured in tissue culture media during a six day period at 24 hour intervals following treatment with 10ng/ml of IL-1β combined with individual treatments with 10 ng/ml of G-CSF, GM-CSF, M-CSF and IL-3. Combined treatments using IL-1β and CSFs resulted in a significant (*P<0.05) increase in GAG loss from cartilage explants at days 1 and 4. Significant (*P<0.05) increases in GAGs loss were also observed on days 3 and 4 following treatment with GM-CSF and IL-1β, and on day 4 following treatment with IL-1β and IL-3.

Combined treatments of CSFs and IL-1β resulted in a trend of increased GAG concentrations detected in the media compared to respective controls. However, significant increases (*$P<0.05$) in all combined treatments were only detected on days 1 and 6. Combined treatments with IL-1β and GM-CSF, and IL-1β and IL-3, caused significant increases in GAG release from the cartilage explants on days 3 and 4, and day 3, respectively. The combined treatment regime with IL-1β and CSFs (Fig. 7.1.7) produced results that were comparable to treatment using IL-1β in the co-culture system on days 1 and 6 (Fig 7.1.1). The concentrations of nitrite produced by combined treatment with IL-1β and CSFs were also measured during a 6 day time course. The results from this study were illustrated in Fig. 7.1.8.

7.1.8: Effect of IL-1β and CSFs on nitrite produced by a co-culture of rat cartilage explants with Swiss 3T3 fibroblasts

Fig. 7.1.8: Effect of IL-1β and CSFs on nitrite produced by a co-culture of rat cartilage explants with Swiss 3T3 fibroblasts

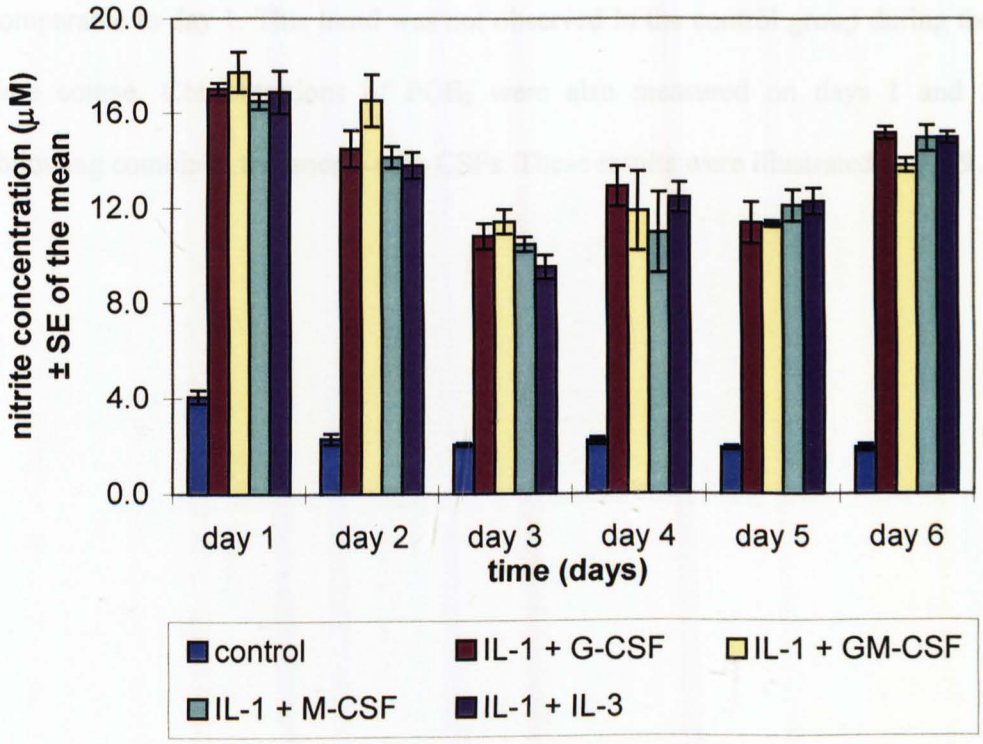


Fig. 7.1.8: A time course of nitrite production by rat femoral head cartilage explants co-cultured with Swiss 3T3 fibroblasts was measured in tissue culture supernatant was measured during a six day period at 24 hour intervals following treatment with 10 ng/ml of IL-1β combined with G-CSF, GM-CSF, M-CSF and IL-3. Analysis of data using a Students t test showed that nitrite concentrations in the culture media were significantly ($p<0.001$) elevated in all combined IL-1β and CSF treated groups compared to respective time control groups at all time points.

Combined treatment with IL-1β amd CSFs resulted in elevated concentrations of nitrite in the media of all groups compared to controls. In all treatment groups nitrite concentrations were initially elevated compared controls followed by a decrease where lowest levels were detected at day 3. Levels of nitrite then increased during the time course reaching elevated levels on day 6 that were comparable to day 1. This trend was not observed in the control group during the time course. Concentrations of PGE_2 were also measured on days 1 and 2 following combined treatments with CSFs. These results were illustrated in 7.1.9.

7.1.9: Effect of IL-1β and CSFs on concentration of PGE_2 produced by a co-culture of rat cartilage explants with Swiss 3T3 fibroblasts

Fig. 7.19: Effect of IL-1β and G-CSF on PGE2 produced by a co-culture of rat cartilage explants with Swiss 3T3 fibroblasts

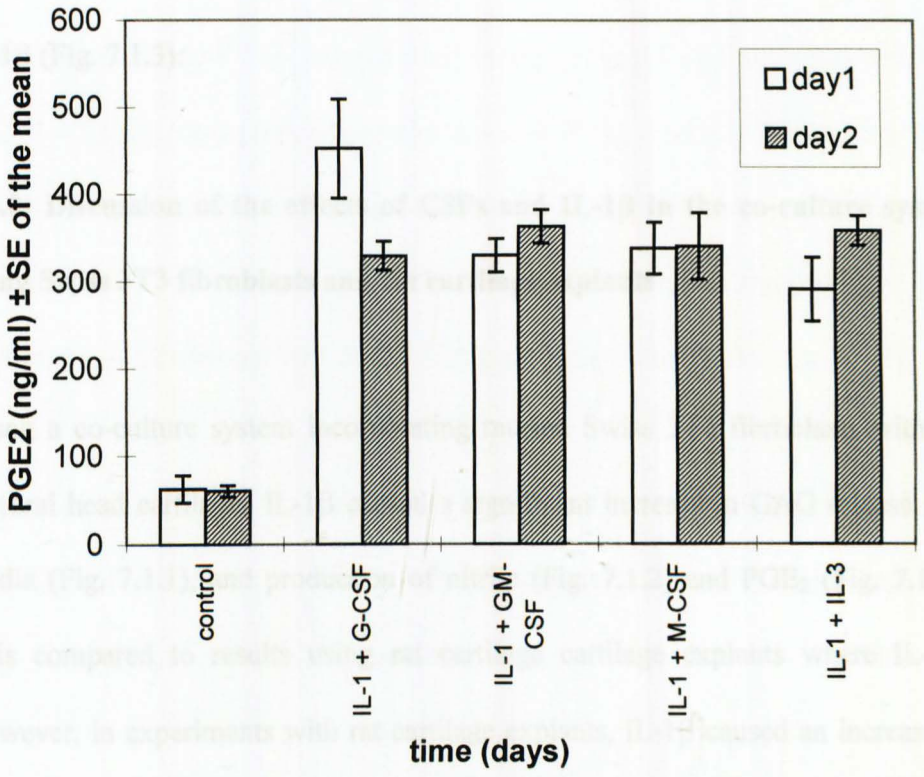


Fig. 7.1.9: Rat femoral head cartilage explants (n=6) co-cultured with Swiss 3T3 fibroblasts were treated with 10ng/ml of IL-1β combined individual treatments with G-CSF, GM-CSF, M-CSF and IL-3 for a 2 day incubation period with a change of media at 48 hours. PGE_2 concentration was measured in the culture media and compared to controls. Analysis of data using a Students t test showed that PGE_2 concentrations in the culture media were significantly ($p<0.001$) elevated in all combined IL-1β and CSF treated groups compared to respective time control groups at all time points.

Concentrations of PGE_2 produced by combined treatments of IL-1β and CSFs in the co-culture system were elevated in all treatment groups at days 1 and 2 compared to controls (Fig. 7.1.9). The concentrations of PGE_2 produced in the co-culture system following combined treatment with IL-1β and CSF were comparable in magnitude to concentrations of PGE_2 produced by treatment with IL-1β (Fig. 7.1.3).

7.2.0: Discussion of the effects of CSFs and IL-1β in the co-culture system using Swiss 3T3 fibroblasts and rat cartilage explants

Uisng a co-culture system incorporating murine Swiss 3T3 fibroblasts with rat femoral head cartilages IL-1β caused a significant increase in GAG release into media (Fig. 7.1.1), and production of nitrite (Fig. 7.1.2) and PGE_2 (Fig. 7.1.3). This compared to results using rat cartilage cartilage explants where IL-1β. However, in experiments with rat cartilage explants, IL-1β caused an increase in production of nitrite (Fig. 3.1.4) and PGE_2 (Fig. 3.1.6). Previous experiments with swiss 3T3 fibroblasts also demonstrated that IL-1β increased production of nitrite (Fig 6.1.2) and PGE_2 (Fig. 6.1.3). It was concluded that the presence of fibroblasts in the system served to augment IL-1β induced loss of GAGs from rat cartilage explants in this *in vitro* model.

Loss of GAGs in the co-culture system occured when concentrations of nitrite (Fig. 7.1.2) and PGE_2 (Fig 7.1.3) were elevated in the culture media. Nitrite

concentrations in the co-culture system (Fig 7.1.2) were elevated compared to nitrite concentrations detected in cartilage explant cultures, following treatment with IL-1β (Fig. 3.1.5). In the co-culture system the maximum concentration of nitrite detected was 14.6 +/- 1.5 μM on day 2 (Fig 7.1.2), compared to previous experiments with rat cartilage explants where the maximum concentration of nitrite detected was 7.3 +/- 1.0 μM, also on day 2 (Fig 3.1.5). Fibroblasts produced 13.2 +/- 2.3 μM nitrite following treatment with IL-1β on day 2 (Fig. 6.1.2). It was concluded that cartilage explants in the co-culture system were exposed to elevated levels of nitrite (Fig 7.1.2) compared to previous experiments using cartilage explants treated with IL-1β during a six day time course (Fig. 3.1.5).

PGE_2 concentrations in the co-culture system (Fig 7.1.3) were elevated compared to PGE_2 concentrations detected in cartilage explant cultures, following treatment with IL-1β (Fig. 3.1.6). In the co-culture system the maximum concentration of PGE_2 detected was 378 +/- 50 ng/ml on day 1 (Fig 7.1.3), compared to previous experiments with rat cartilage explants where the maximum concentration of PGE_2 detected was 2.47 +/- 0.17 ng/ml, on day 1 (Fig 3.1.5). Fibroblasts produced 359 +/- 22 ng/ml PGE_2 following treatment with IL-1β on day 2 (Fig. 6.1.3). It was concluded that cartilage explants in the co-culture system were exposed to elevated levels of PGE_2 (Fig 7.1.3) compared to previous experiments using cartilage explants treated with IL-1β during a six day time course (Fig. 3.1.6). It was also concluded that the majority of PGE_2 produced in the co-culture system was derived from fibroblasts.

CSFs were also used in the co-culture system to determine their effects on GAG loss from cartilage explants and effects on nitrite and PGE_2 production. IL-3 produced increased GAG loss at days 1 and 4 from cartilage explants in the co-culture system during a 6 day time course (Fig 7.1.4). This compared to previous cartilage explant experiments where IL-3 did not change GAG release into the media (Fig 4.2.5).

G-CSF, GM-CSF, M-CSF and IL-3 did not affect production of nitrite in the co-culture system (Fig 7.1.5) during a six day time course. This compared to previous experiments with cartilage (sections 4.1.4, 4.1.8, 4.2.2 and 4.2.6) where no change in nitrite production was observed following treatment with G-CSF, GM-CSF, M-CSF and IL-3, respectively. In previous experiments using fibroblasts (section 6.1.6) the aforementioned CSFs did not affect nitrite concentrations during a six day time course. Conversely, G-CSF, GM-CSF and IL-3 caused an increase in production of PGE_2 in the co-culture system. In contast, M-CSF did not affect the production of PGE_2 in the co-culture system. This compared to previous experiments with fibroblasts where G-CSF, GM-CSF, M-CSF and IL-3 did not affect production of PGE_2 (section 6.1.6). However, previous experiments using cartilage explants demonstrated that treatments with G-CSF (Fig. 4.1.5) and IL-3 (Fig 4.2.7) caused an increase in PGE_2 production, yet GM-CSF did not affect PGE_2 levels. The elevated concentration of PGE_2 produced by G-CSF (Fig. 4.15) and IL-3 (Fig 4.2.7) in cartilage experiments were 4.16 +/- 1.10 and 10.60 +/- 0.31 ng/ml, respectively. This compared to elevated PGE_2 concentrations produced by G-CSF, IL-3 and GM-CSF in the co-culture system that were 215 +/-

76, 256 +/- 83 and 177 +/- 21 ng/ml respectively. It was concluded from these results that the presence of fibroblasts in the co-culture system serves to augment the production PGE_2 in the presence of G-CSF, GM-CSF and IL-3.

Combined treatments using CSFs and IL-1β were used, as in previous experiments using fibroblasts (chapter 6) and cartilage explants (chapter 5) to determine the effects on GAGs, nitrite and PGE_2 in the co-culture system. Combined treatments with IL-1β and all CSFs caused increased production of nitrite (Fig 7.1.8) and PGE_2 (Fig. 7.1.9) in the culture media. However, these effects were comparable increases in nitrite (Fig. 7.1.2) and PGE_2 (Fig. 7.1.3) production observed following treatment with IL-1β in the co-culture system.

Il-1β was shown regulate the loss of GAGs in the fibroblast-cartilage co-culture system and to increase production of nitrite and PGE_2 in this system. A study was performed using IL-1β as a positive control and attempting to elucidate the involvement of intracellular signal transduction pathways in the Swiss 3T3 fibroblasts and rat cartilage explants leading to production and release of nitrite into the culture media studying cartilage and fibroblasts in separate experiments. These results were illustrated in chapter 8.

Chapter 8:

Intracellular Signaling pathways and release of NO: Effects of MAP kinase inhibitors in Swiss 3T3 fibroblasts and rat femoral head cartilage.

8.1.0 Introduction

Previous studies have shown that IL-1β produces elevated concentrations of NO and PGE_2 in both rat cartilage explants (chapter 3) and Swiss 3T3 fibroblasts (chapter 6). Elevated concentrations of NO and PGE_2 in a co-culture system have correlated with elevated release of GAGs from the cartilage matrix (chapter 7) in a fibroblast-cartilage co-culture system. However, the intracellular events from IL-1β binding to the cell to release of NO in rat chondrocytes and Swiss 3T3 fibroblasts have not been fully characterised. It has been shown that intracellular signaling pathways leading to production of NO are activated by a number of factors such as cytokines, endotoxins and stress. Nitric oxide was the focus of this study since it has been implicated with GAG breakdown (Hassan *et al.*, 1998), activation of metalloproteinases (Murrell *et al.*, 1995), induction of COX activity (Honda *et al.*, 2000) and induction of PGE_2 production (Blanco and Lotz, 1995). It has been shown in studies by (Badger et *al.*, 1998; Ridley et *al.*, 1998) that IL-1 induced iNOS expression and COX-2 mRNA were downregulated respectively, when the p38 MAP kinase pathway was specifically inhibited.

Two MAP kinase pathways, p38 MAP kinase and ERK 1/2 were targetted using highly specific inhibitors to determine whether production of NO induced by IL-1β in fibroblasts and rat cartilage explants could be suppressed. These MAP kinases were selected on the basis that both p38 mitogen activated protein (MAP) kinase and extracellular signal related kinase (ERK) can act as intermediates in cytokine and growth factor signalling within mammalian cells (Cohen, 1997)

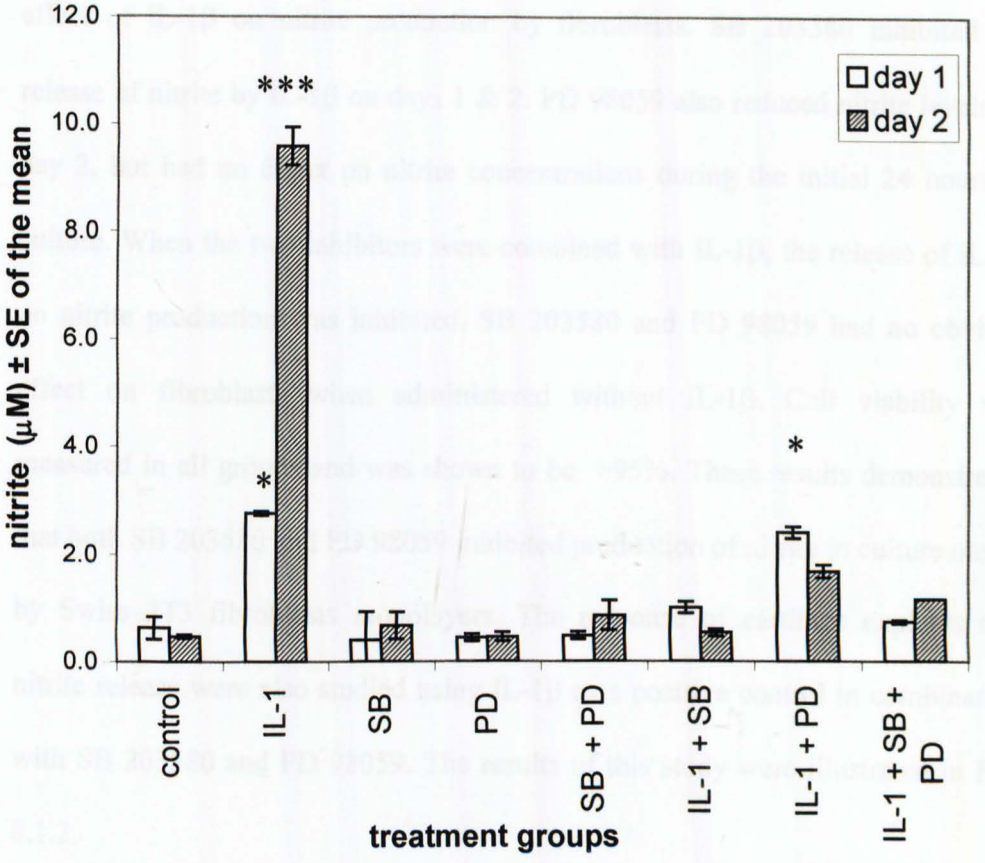
(Wang *et al.*, 1998). A study by Alessi *et al.* (1995) showed that PD 98059 was a specific inhibitor of ERK 1 and ERK 2 ligands within Swiss 3T3 cells and human fibroblasts. A study by Badger *et al.* (1998) using bovine chondrocyte cultures and bovine explant cultures demonstrated that SB 203580 inhibits p38 MAP kinase, iNOS expression and production of nitric oxide. Both SB 203580 and PD 98059 were therefore used to determine if they inhibited nitrite induced by rat cartilage explants and Swiss 3T3 Fibroblasts.

SB 203580 was used at a concentration of 2μM. The IC_{50} value of nitrite inhibition following treatment with IL-1β was characterised by Badger et al., (1998) using bovine chondrocytes was 0.6 - 1.0 μM. In the present study, SB 203580 was used at a concentration of 2μM because it has been shown that SB 203580 remains a specific inhibitor of p38 MAP kinase affecting activity of ERK at concentrations lower than 10μM. PD 98059 was used at a concentration of 50μM. This concentration was used since Alessi *et al.* (1995) showed that the IC_{50} for inhibition of ERK 1 / ERK 2 at this concentration was 50μM. Fibroblast viability was measured following experiments using SB 203580 and PD 98059 to confirm that these inhibitor were not causing cell death at these concentrations. IL-1β was used at 10 ng/ml as a positive control for nitrite production in both cartilage and fibroblast experiments. A maximum final concentration of 0.5% v/v DMSO was used as a vehicle for these inhibitors. DMSO controls were run to ensure that toxicity was not induced by the vehicle and DMSO-IL-1β controls

were run to ensure that DMSO did not reduce nitrite concentrations in the media. The results of these experiments are shown in Figs 8.1.1 & 8.1.2.

Fig. 8.1.1: The effect of SB 203580 and PD 98059 on nitrite production induced by IL-1β in Swiss 3T3 fibroblasts.

Fig: 8.1.1: Effect of SB 203580 and PD 98059 inhibitors on IL-1β induction of nitric oxide in Swiss 3T3 fibroblasts



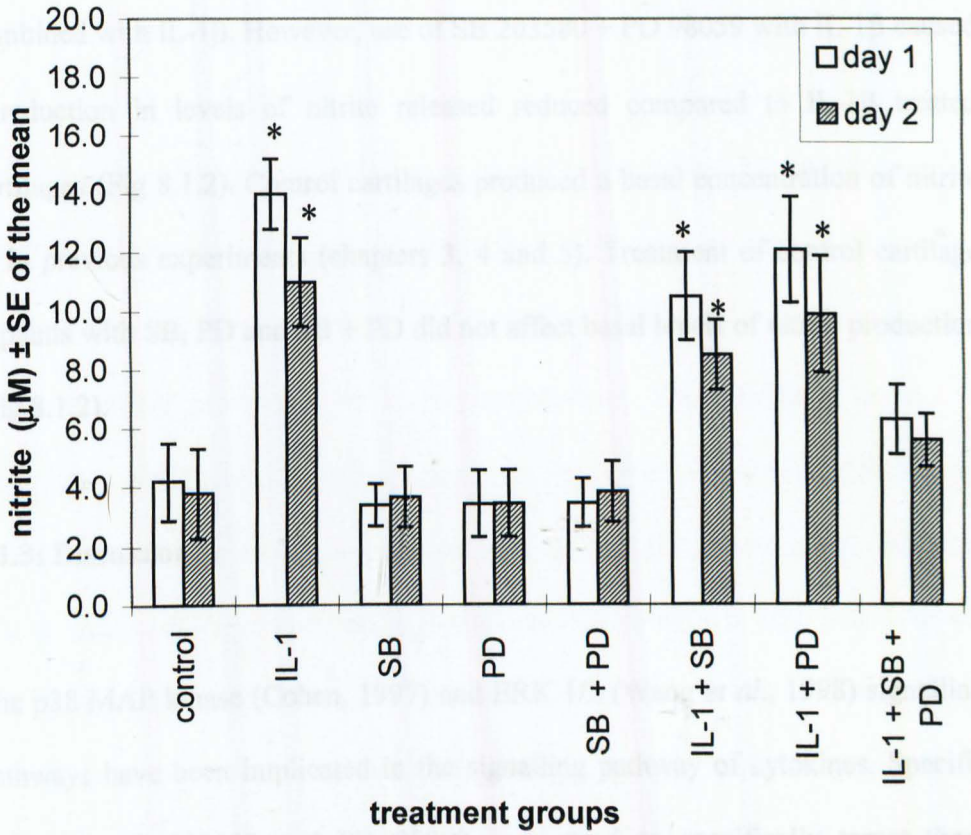
8.1.1: Swiss 3T3 Fibroblasts were treated with 10ng/ml IL-1β, 2μM SB 203580 (SB), 50μM PD 98059 (PD), IL-1β+SB, IL-1β+PD and IL-1β+SB+PD for a 2 day period with a change of media following the initial 24 hours. Analysis of data using a Students t test showed that nitrite concentrations in the culture media were significantly (*$P<0.05$) elevated in IL-1β and IL-1β + PD treated groups points on day 1 and significantly elevated (*$P<0.001$) by IL-1β on day 2, compared to respective control groups. The effect of IL-1β on nitrite was suppressed by SB on

days 1 and both SB and PD on day 2. The combined treatment with SB + PD also suppressed nitrite production on days 1 and 2.

IL-1β caused an increase in nitrite production by fibroblasts as in previous experiments (Fig 6.1.2). SB 203580 and PD 98059 both caused a reduction in the effect of IL-1β on nitrite production by fibroblasts. SB 203580 inhibited the release of nitrite by IL-1β on days 1 & 2. PD 98059 also reduced nitrite levels on day 2, but had no effect on nitrite concentrations during the initial 24 hours of culture. When the two inhibitors were combined with IL-1β, the release of IL-1β on nitrite production was inhibited. SB 203580 and PD 98059 had no obvious effect on fibroblasts when administered without IL-1β. Cell viability was measured in all groups and was shown to be >95%. These results demonstrated that both SB 203580 and PD 98059 inhibited production of nitrite in culture media by Swiss 3T3 fibroblasts monolayers. The response of cartilage explants and nitrite release were also studied using IL-1β as a positive control in combination with SB 203580 and PD 98059. The results of this study were illustrated in Fig. 8.1.2.

8.1.2: The effect of SB 203580 and PD 98059 on nitrite production induced by IL-1β in rat cartilage explants.

Fig 8.1.2: Effect of SB 203580 and PD 98059 on IL-1β induction of nitric oxide in rat cartilage explants



Graph 8.1.2: Rat cartilage explants were treated with 10ng/ml IL-1β, 2μM SB 203580 (SB), 50μM PD 98059 (PD), IL-1β+SB, IL-1β+PD and IL-1β+SB+PD for a 2 day period with a change of media following the initial 24 hours. Analysis of data using a Students t test showed that nitrite concentrations in the culture media were significantly (*$P<0.05$) elevated in IL-1β,IL-1β + PD and IL-1β + SB treated groups points on days 1 and 2. The effect of IL-1β on nitrite production was ameliorated by SB + PD on days 1 and 2. SB, PD and SB + PD did not change nitrite concentrations compared to same time controls.

Results shown in Fig. 8.1.2 show the effects of SB 203580 and PD 98059 when combined with IL-1β. In this study IL-1β caused increased production of nitrite by rat cartilage explants. However, unlike the comparative study using fibroblasts (Fig. 8.1.1), concentrations of nitrite produced by cartilage explants were not significantly reduced by separate treatments with SB 203580 and PD 98059 when combined with IL-1β. However, use of SB 203580 + PD 98059 with IL-1β caused a reduction in levels of nitrite released reduced compared to IL-1β treated cartilages (Fig 8.1.2). Control cartilages produced a basal concentration of nitrite as in previous experiments (chapters 3, 4 and 5). Treatment of control cartilage explants with SB, PD and SB + PD did not affect basal levels of nitrite production (Fig 8.1.2).

8.1.3: Discussion

The p38 MAP kinase (Cohen, 1997) and ERK 1/2 (Wang *et al.*, 1998) signalling pathways have been implicated in the signalling pathway of cytokines. Specific inhibitors SB 203580 and PD 98059 were used to specifically target these respective intermediates to determine if nitrite production induced by IL-1β would be suppressed. A study using Swiss 3T3 fibroblasts (Fig. 8.1.1) demonstrated that SB 203580 and PD 98059 inhibited production of nitrite induced by IL-1β. This compared with a study by Da Silva *et al.*, (1997) using murine astrocytes that showed that blockade of p38 MAP kinase inhibited iNOS expression induced by IL-1β, however, PD 98059 had no effect on iNOS expression induced by IL-1β.

The different cell types, murine astrocytes and 3T3 fibroblasts, may account for the differences observed with regard to PD 98059 inhibition of iNOS expression in the respective studies. It was concluded from the results of the present study that both p38 MAP kinase and ERK 1/2 pathways are involved in transduction of the intracellular signal induced by IL-1β that results in nitrite production by Swiss 3T3 fibroblasts.

However, in a comparative study using rat cartilage explants SB 203580 and PD 98059 failed to suppress the effects of IL-1β on nitrite production (Fig 8.1.2) in rat cartilage explants. However, when SB 203580 and PD 98059 were combined the effect of nitrite production by IL-1β was reduced. These findings are not in accordance with those in a study by Badger *et al.*, (1998) where SB 203580 alone, at comparative concentrations to those used within the present study, inhibited nitrite produced by bovine cartilage explants. Conversely, a study by Caivano (1998) showed that SB 203580 did not affect induction of iNOS in macrophages. The discrepancy between results obtained in the present study compared to the study by Badger *et al.*, (1998) and Caivano, (1998) may be due to the differences between different cell and species types. Results obtained by Badger *et al.*, (1998) supported data obtained in this study showing that PD 90859 and SB 203580 do not influence constitutive production of nitrite by cartilage explants. It was concluded that both SB 203580 and PD 98059 were involved in the inducible nitrite production pathway in present study and therefore p38 MAP kinase and ERK 1 / 2 are involved in transduction of the intracellular signal, induced by IL-1β, leading to production of nitrite by rat cartilage explants. It was speculated that

the lack of effect of SB 203580 alone on cartilage explants may have been caused by restriction of SB 203580 interaction with chondrocytes by the rat cartilage matrix.

It was planned to conduct experiments with these inhibitors to measure their effect on PGE_2 production by fibroblasts and cartilage explants and their effect in the co-culture system where fibroblasts and cartilage produced significant release of nitrite, PGE_2 and GAGs from the cartilage matrix following treatment with IL-1β. However, it was not possible to perform this study due to limited rat cartilage availability.

Chapter 9:

General Discusssion

9.1.0: Cartilage explants used as a model of cartilage breakdown

Cartilage explants have been previously utilised to model the breakdown of articular cartilage *in vitro*. The preliminary study in this research involved a study of GAG loss from cartilage explant in an *in vitro* system (section 3.1.0). The aim of this study was to select a cartilage explant type that would be suitable for investigating the process of cartilage breakdown by measurement of glycosaminoglycans (GAGs), production of nitric oxide (NO) and prostaglandinE_2 (PGE_2) in response to cytokines. GAGs have been shown to be essential components of cartilage matrix and are lost from the extracellular matrix as a consequence of cartilage breakdown, (Billington *et al.*, 1998; Bottomley *et al.*, 1997; Wertheimer *et al.*, 1995). NO (Evans and Stefanovicic, 1996) and PGE_2 (Prete *et al.*, 1997) are both markers of inflammation that may serve to exacerbate the loss of GAGs from the cartilage matrix.

The criteria for selection of a cartilage type was based upon the activity of cartilage under control conditions with respect to levels of GAGs in cartilage explants and culture media during a 6 day time course. It was considered important that cartilage components were not lost under control conditions in an *in vitro* culture environment. This characteristic was paramount to ensure that cartilage breakdown could be measured as a consequence of cytokine treatment and distinguished from cartilage breakdown caused as a consequence of the *in*

vitro conditions. Availability of cartilage samples and uniformity of cartilage explant samples were also considered when this pilot study was undertaken.

Measurement of GAGs in media and explants following explant culture revealed that both human and rat cartilage displayed suitable characterisitics for use an *in vitro* study. Rat and human cartilage explants lost 20% and 27%, respectively, of GAG content during culture over a 6 day period. Conversely, porcine nasal and articular cartilage explants showed high percentage GAG losses into the culture media during the 6 day incubation. Porcine nasal and porcine articular cartilage lost 46% and 43% of total GAG content as a consequence of explant culture during 6 days. Although human tissue samples produced a suitable profile with respect to GAG loss under control conditions, the availability of human cartilage explants was limited. The quantity of human femoral head cartilage was variable on human femoral heads obtained from hip fracture patients. The process of extraction of human cartilage also led to regional variability in cartilage samples since articular cartilage is composed of different zones with varying physical properties (section 1.1.5).

Unlike human cartilage explants, rat cartilage explants consisted of entire cartilage femoral heads comprising all of the zonal regions described. It was therefore considered that entire rat cartilage explants would provide more reliable data in a model of cartilage breakdown than using explants derived from different regions of human femoral head samples.

Further studies demonstrated that rat femoral head cartilages were responsive to inflammatory mediators such as interleukin-1β. Treatment of rat cartilages with 10 ng/ml of IL-1β resulted in generation of NO and PGE_2. The response of rat cartilage explants to IL-1β produced a standard for comparison in successive studies using colony stimulating factors (CSFs).

It was surprising that neither IL-1β, TNF-α or LPS induced an increase in loss of GAGs from the explants during explant culture. In other studies IL-1β (Seed *et al.*, 1993; Hanglow *et al.*, 1995; Spirito *et al.*, 1995; Stefanovic-Racic *et al.*, 1997) TNF-α (Homandberg *et al.*, 1998; Stichtenoth and Frolich, 1998) and LPS (Morales *et al.*, 1984) have all been shown to increase cartilage breakdown. Indeed, the use of IL-1β and TNF-α antagonists have been implicated as a therapeutic aid to reduce the symptoms associated with arthritic disease (Moreland *et al.*, 1997). Despite the effects of IL-1β on NO and PGE_2 production, the rat cartilage explants failed to show increased loss of GAGs in this system following treatment with IL-1β. The reason for this may have been due to the fact that rat tissue does not produce MMP-1, as subsequently indicated in a study by (Greenwald *et al.*, 1998). It was speculated that the ability of cytokines to interact with chondrocytes may have been hindered by the cartilage matrix in the explant experiments performed in this study. It was also noted that the source of recombinant cytokines was different from that of cytokines used in other cited studies. These factors may have influenced the ability of rat chondrocytes to respond to cytokines in this study. The effect of cartilage breakdown in rat models

of arthritis may also rely upon interaction with other moieties, such as synovium cells, that were not present within the *in vitro* culture system used in the current study (chapters 3, 4 and 5). Indeed, studies performed in chapter 7 showed that introduction of fibroblast cells into the culture system greatly enhanced release of GAGs following addition of cytokines to fibroblast-cartilage co-cultures (discussed in section 9.1.4).

Other studies have shown that aggrecanase activity may be indepedent of IL-1β in bovine cartilage (Ilic *et al.*, 2000). However, aggrecanase activity in rat cartilage has not been confirmed to date. Lack of aggrecanase activity in rat cartilage may be another factor resulting in the lack of response to IL-1β in the current study. Control conditions and IL-1β treated cartilages used to produce results in chapter 3 were compared with the responses generated by CSFs (chapter 4).

9.1.1: The effects of CSFs on rat cartilage explants.

CSFs have been detected in the synovial joint and have been shown to be produced by cartilage chondrocytes (Campbell *et al.*, 1991 and 1993) and tissues surrounding the synovial joint (Hamilton *et al.*, 1993). However, the effects of CSFs on cartilage explants in an *in vitro* system have not been previously studied. The rat cartilage explant system was used to measure the effects of CSFs with respect to GAGs, NO and PGE_2.

It was interesting to note that G-CSF reduced GAG concentrations detected in cartilage explants compared to controls, yet did not increase release of GAGS into the cutlure media. Like G-CSF, IL-6 has been described by van de Loo *et al.*, (1997) as a cytokine that reduces GAG concentrations in articular cartilage. Analysis of the structure of IL-6 has revealed that it has comprises a four domain alpha helical structure (Appendices II) (Somers *et al.*, 1997). Comparison with the structure of G-CSF has shown that it also has a similar structure (Zinc *et al.*, 1994), (Appendices III).

It has been shown by (Taga and Kishimoto, 1997) that IL-6 binds to a gp130 that is a common component in the IL-6 family of cytokine signalling pathway and that the G-CSF receptor has a high percentage homology was the gp 130 subunit of the IL-6 receptor complex. IL-6 binds to the IL-6 receptor complex which in turn activates the signal transducer and activator of transcription 3 (STAT3) factor. G-CSF, like IL-6, also activates the STAT 3 pathway in l. STAT 3 is translocated to the cell nucleus where it activates various transcription factors, including CCATT enhancer binding protein (C/EBP) (Chen *et al.*, 1999). The C/EBP family of proteins have been associated with signal transduction pathways initiated by both IL-1 and IL-6 and C/EBP proteins have been demonstrated to interact with nuclear factor-κB (NF-κB) proteins (Xia, 1997). It is therefore possible that G-CSF may influence GAG concentrations and nitrite production in cartilage explants via similar mechanism to those described with regard to IL-6 signaling.

Unlike G-CSF, GM-CSF and M-CSF did not influence the role of chondrocytes in rat cartilage explants with respect to GAG concentration, and nitrite and PGE_2 production. However, IL-3 induced a significant increase in production of PGE_2 by cartilage explants in the culture media. These findings suggest that both G-CSF and IL-3 influence rat cartilage chondrocytes within a cartilage matrix. It has been suggested that both PGE_2 and NO may compromise GAG stability in the cartilage matrix directly, (Hassan *et al.*, 1998; Prete *et al.*, 1997). Alternatively, CSFs may activate transcription factors such as C/EBP and NF-κB that may initiate transcription and production of other inflammatory mediators such as cytokines, metalloproteinases, reactive oxidative species and prostaglandins. Endogenous production of inflammatory mediators by cartilage explants may then increase the potential for cartilage breakdown. In disease pathology associated with arthritic disease this effect may serve to increase migration of immune cells toward articular cartilage surfaces and increase their potential to produce further pro-inflammatory cytokines, metalloproteinases, ROS and prostaglandins. This effect in turn may drive the destructive process and produce a self perpetuating circle of events that would promote loss of GAGs from the cartilage matrix. Loss of GAGs from the matrix would decrease the hydration of cartilage and its concordant abillity to maintain integrity under compressive forces resulting in loss of structure and consequential loss of function.

9.1.2: The combined effects of CSFs with IL-1β on rat cartilage explants.

Studies by Campbell *et al.* (1991; 1993) revealed that cartilage explants produce CSFs in response to IL-1β. It is therefore possible that CSFs and IL-1β may be located in the proximity of articular cartilage in a combined presence during arthitic disease. Cartilage explant experiments in this study revealed that combined treatment with IL-1β and G-CSF caused increased loss of GAGs into the culture media. This compared to separate treatments with IL-1β (Fig 3.13) and G-CSF (section 4.12), where GAG concentrations in the media were unaffected. This showed that the combined effect of these cytokines had a greater potential to reduce GAG concentrations in explants compared to separate treatments. PGE_2 concentrations were also assessed following combined treatments with IL-1β and CSFs (chapter 5).

PGE_2 concentrations were increased by IL-3 and IL-1β (Fig 5.27) in the combined study. This was not surprising since IL-1β (Fig 3.16) and IL-3 (Fig. 4.27) promoted production of PGE_2 as separate treatments. Unlike IL-3, GM-CSF did not cause cartilage explants to produce elevated concentrations of PGE_2 in the culture media (section 4.19). However, PGE_2 concentrations were increased in culture media when combined treatments of GM-CSF and IL-1β were administered to cartilage explants (Fig. 5.1.8). These experiments suggested that both IL-3 and GM-CSF had a synergistic effect with IL-1β with regard to PGE_2 release from rat cartilage explants. This was interesting since both IL-3 and

GM-CSF signal through an identical β-chain in the receptor complex (Mulhern *et al.*, 2000). These cytokines initially bind to a cytokine specific α-chain prior to recruitment of the common β-chain in order to transduce signals within the cell. The β-chain consists of an intracellular domain containing eight phosphorylatible tyrosine residues that are required for JAK-STAT signalling and an extracellular domain containing sequence features that are restricted to cytokine receptors. Both IL-3 and GM-CSF have been demonstrated to have an overlapping hierachy of function in hematopoietic cells, (Nimer and Uchida, 1995). It is therefore speculated that GM-CSF and IL-3 may contribute to PGE_2 production *in vivo* since the there is evidence for GM-CSF receptor expression in both RA and OA synovial tissue (Berenbaum *et al.*, 1994).

Experiments in the present study showed that IL-1β, TNF-α (chapter 3) and CSFs interact with cartilage in an *in vitro* environment and induce release of NO and PGE_2. In an *in vivo* environment these effects would be integrated by interactions with other cell types. The synovial joint membrane, which under normal physiology is relatively acellular, is infiltrated in RA pathology by T cells, macrophages and plasma cells (Odeh, 1997). In rheumatoid arthritis the hyper proliferation of the synovium results in an invasive pannus tissue composed of fibroblast-like cells growing toward the surface of articular cartilage within synovial joints (Edwards, 1995). In a further study the intention was to extend the cartilage explant model to incorporate a fibroblast cell type. Fibroblasts were chosen to culture with cartilage explants to determine if the effects of IL-1β and

CSFs would be enhanced or diminished by their presence. Swiss 3T3 fibroblasts were intially used in a pilot study to determine if they released GAGs, NO or PGE_2 in response to culture with inflammatory cytokines (chapter 6).

9.1.3: Fibroblast monolayers and their response to inflammatory cytokines.

Swiss 3T3 fibroblasts were initially cultured in control media identical to that used in cartilage explant studies. This provided a basis for studying fibroblasts activity in conditions that could be repeated using a co-culture system in following experiments. Fibroblasts in control conditions produced a basal level of PGE_2 in the absence of IL-1β, however, NO and GAGs were not detected in the culture media. Swiss 3T3 fibroblasts have been shown to possess GAG like moieties on the surface of the cell membrane (Tsiganos *et al.* 1982), however GAGs were not detected in media from any experiments involving fibroblast monolayers (chapter 6).

Treatment with TNFα caused an increased production of NO (6.1.4) and PGE_2 (6.1.5) by Swiss 3T3 fibroblasts. Treatment with IL-1β also caused an increased production of NO (Fig 6.1.2) and PGE_2 (Fig 6.1.3) by Swiss 3T3 fibroblasts. This was anticipated since IL-1 has been shown to induce PGE_2 and NO production by Swiss 3T3 cells (Burch *et al.*, 1989). CSFs were also applied to fibroblasts in order to determine if they increased the production of the previously measured parameters, however, no changes in production of NO and PGE_2 were observed

(section 6.1.6). Combined treatments with IL-1β and CSFs produced increased levels of NO and PGE_2 compared to controls. The objectives were achieved since it was shown that Swiss 3T3 fibroblast did not release GAGs into the culture media and that Swiss 3T3 fibroblasts were responsive to IL-1β treatment. Swiss 3T3 fibroblasts were then used in fibroblast-cartilage co-culture experiments (chapter 7).

9.1.4 Cartilage-fibroblast culture as a model of cartilage breakdown.

Integration of Swiss 3T3 fibroblasts and rat femoral head cartilages (chapter 7) produced results that were markedly different from previous experiments (chapters 3, 4 and 5) with regard to GAG release from cartilage explants. Addition of IL-1β to the system caused an increased release of GAGs (Fig 7.11) from the cartilage explants and elevated levels of nitrite (Fig 7.12) and PGE_2 (Fig 7.13) in the culture media compared to control fibroblast-cartilage co-cultures. These results demonstrated that fibroblasts co-cultured with cartilage explants caused increased loss of GAGs from cartilage explants and that this process was increased in magnitude by IL-1β. It was assumed that all GAGs detected in the media were derived from cartilage explants and not fibroblasts, since GAGs were not detected in either control or cytokine treated fibroblast media (chapter 6). In an *in vivo* scenario fibroblasts may influence breakdown of the cartilage matrix in normal physiology and in RA due to interactions with infiltrating cells (Odeh, 1997) and synovial fibroblasts (Edwards, 1995).

A recent study showed that CSFs may also increase the processes leading to cartilage destruction *in vivo*. It was postulated that a local network of CSFs may exist that orchestrate the cellular interactions and resulting tissue damage in arthritic lesions (Campbell *et al.*, 2000). In a murine model of joint inflammation it was demonstrated that systemic exogenous IL-1β, M-CSF and GM-CSF all increased synovitis, i.e. proliferation of the synovium, infiltration of inflammatory cells into blood vessels around the synovium.

In the present study the effects of CSFs were examined with regard to GAGs lost from cartilage and production of NO and PGE_2 in the co-culture system. The objective of this study was to determine if exogenous CSFs had a direct effect on the level of GAGs released from cartilage explants in the presence of fibroblasts, as observed with IL-1β.

Treatment of co-cultures with IL-3 caused an increased loss of GAGs from cartilage explants on days 1 and 4 (Fig 7.1.4). Conversely, addition of G-CSF, GM-CSF, M-CSF to fibroblast-cartilage co-cultures did not influence GAG loss from cartilage explants (Fig. 7.1.4). Individual treatments with CSFs did not increase nitrite concentrations (Fig. 7.1.5) in co-cultures. This was surprising since G-CSF increased nitrite production in cartilage explants (Fig 4.1.3). Levels of PGE_2 were elevated by G-CSF, GM-CSF, IL-3 in the co-culture system, but not by M-CSF (Fig. 7.1.6).

Treatments with CSFs combined with IL-1β were also used in the co-culture system. The observed effects of combined treatment with CSFs and IL-1β resembled those observed with IL-1β. GAG concentrations in the media of combined treatment groups showed a trend of being elevated compared to controls (Fig 7.1.7), although no single combination treatment appeared to be elevated compared to other combination treatment groups or treatments with IL-1β (Fig 7.1.1). Nitrite (Fig 7.1.8) and PGE_2 (Fig 7.1.9) levels were elevated in all combined treatment groups compared to controls. Elevated nitrite levels in all combined IL-1β and CSF treated groups (Fig 7.1.8) compared to IL-1β induced nitrite concentrations (Fig. 7.1.2) in co-cultures. Elevated PGE_2 levels in all combined IL-1β and CSF treated groups (Fig 7.1.9) compared to IL-1β induced PGE_2 concentrations (Fig. 7.1.3) in co-cultures. These results suggest that CSFs combined with IL-1β did not affect GAG loss (Fig. 7.1.7), nitrite production (Fig. 7.1.8) and PGE_2 production (Fig. 7.1.9) in this *in vitro* fibroblast cartilage co-culture model. However, the results of this study may support the evidence by Campbell *et al.*, (2000), suggesting that CSFs may increase the synovitis in a mouse model of joint inflammation. Indeed, if PGE_2 production is increased *in vivo*, it may promote pro-inflamatory activity by increasing blood vascular permeabillity (Amin *et al.*, 1997), increase bone resorption (Horton *et al.*, 1999).

Similarly, if NO production is increased *in vivo*, it may promote GAG degradation in the cartilage matrix (Hassan *et al.*, 1998), activation of MMPs (Murrel *et al.*,

1994), induction of COX-2 activity (Honda *et al.*, 2000) and increased synthesis of PGE_2 (Blanco and Lotz, 1995).

It was apparent from this study, using co-cultured fibroblasts and cartilage explants, that G-CSF, GM-CSF and IL-3 increase the production of PGE_2 (Fig. 7.1.6). The concentrations of PGE_2 produced in this study (>200ng/ml) suggested that PGE_2 was derived from fibroblasts. Previous experiments (chapter 6) showed that concentrations of PGE_2 produced by fibroblasts were a magnitude higher than those produced by cartilage explants (chapters 3, 4, and 5). Treatments with CSFs on fibroblast monolayers did not increase PGE_2 production (section 6.1.7). It was speculated that CSFs in the co-culture system may have induced production of inflammatory mediators, such as IL-1β and / or TNFα, by rat cartilage explants. These inflammatory mediators may have then induced production of PGE_2 by fibroblast monolayers in the co-culture system. This hypothesis was considered since GM-CSF increases synthesis of both IL-1β and TNFα in monocyte / macrophage cells (Deresinski and Kemper, 1998).

It was demonstrated in the present study that IL-1β was required to produce increased GAG release from explant matrix and production of NO in the co-culture system. The production of NO was considered with particular interest since it may have direct effect on the cartilage matrix. A study was performed separately on cartilage explants and fibroblasts to examine the effects of intracellular pathways leading to production of NO using specific inhibitors of

elements implicated in mediating IL-1β intracellular signalling. This involved using specific inhibitors to target p38 MAP kinase and ERK 1 / ERK 2 pathways (chapter 8).

9.1.5: Use of specific inhibitors to block p38 MAP kinase and ERK 1 / ERK 2 in fibroblasts and rat cartilage explants.

The research project was concluded by performing a study to determine if p38 MAP kinase and ERK 1/ERK 2 inhibition resulted in inhibition of nitrite production, induced by IL-1β. p38 MAP kinase and ERK 1/2 target were selected and targeted using the specific inhibitors SB 203580 and PD 98059, respectively (Fig. 8.1.1).

Results from experiments conducted on fibroblasts showed that both SB 203580 and PD 98509 inhibited nitrite production induced by IL-1β. It was therefore not surprising to observe that combined treatments with these inhibitors also reduced IL-1β induced nitrite production in fibroblast monolayers. These results demonstrated that production of nitrite in fibroblasts requires the activity of both p38 MAP kinase and PD 98059. These were comparable with a study that showed p38 MAP kinase reduced iNOS production and release of nitrite in bovine chondrocyte monolayers (Badger *et al.*, 1998).

A comparable study was performed on rat cartilage explants to determine if similar effects could be detected in chondrocytes within the cartilage matrix in an *in vitro* system. In contrast to experiments with fibroblast monolayers, SB 203580 and PD 98059 failed to suppress the effect of IL-1β on production. However, IL-1β induced nitrite production was decreased by combined treatment SB 203580 and PD 98059. These results suggested that both p38 and ERK 1/ ERK 2 are components in the IL-1β signalling pathway leading to increased generation of nitric oxide in rat chondrocytes within cartilage explants. It was speculated that the cartilage matrix impeded the effectiveness of these inhibitors to interact with the intracelular targets within chondrocytes.

9.1.6: Summary

The findings from this thesis produced several key conclusions about the use of the cartilage explant system coupled with fibroblasts as an *in vitro* model of cartilage response to cytokines.

Firstly, cartilage is a tissue that is variable in its structure and responsiveness to stimuli depending upon the location and species from which it is derived. It is important to consider the heterogeneity of its structure and standardise the conditions and environment within an *in vitro* system. Secondly, cartilage is a tissue that interacts with its surrounding environment *in vivo*. Use of cartilage explants *in vitro* provides a useful indication of chondrocyte functions (chapters 3, 4 and 5), however, these observed functions may be impeded and modified by

interactions with fibroblasts, as demonstrated by studies using the co-culture system (chapter 7).

In this system, IL-1β has been characterised as a cytokine that increases markers of inflammation by cartilage explants (chapter 3). Although IL-1β did not cause GAG release in rat cartilage explants (chapter 3), the catabolic effects of IL-1β were demonstrated in the co-culture system (chapter 7). The co-culture model has potential use as a system for screening activity of specific compounds that may be designed to reduce GAG loss from the cartilage matrix. There are also many other factors that may be studied in such a system, such as the synthesis of proteoglycans by chondrocytes, the production and secretion of MMPs, chemokines and the activity characterised anti-inflammatory cytokines such as IL-10 and IL-13. It would also make an interesting study to examine the loss of collagenous components from the cartilage extracellular matrix. This would provide further evidence of catabolic activity occuring in the system.

The effects of the CSFs on cartilage explants were also interesting and novel; the study revealed that CSFs may indeed increase the potential of fibroblasts and cartilage to regulate the inflammatory responses. Notably, G-CSF and IL-3 were shown to be involved in the regulation of NO and PGE_2 production (chapter 4), respectively, and G-CSF, GM-CSF and IL-3 increased PGE_2 production when combined with IL-1β and in the co-culture system (chapter 7). The Effects of G-CSF with IL-1β strongly suggest that there may be combined effects resulting

in loss of GAGs and increased production of nitrite by cartilage explants alone (chapter 5). These novel observations show that CSFs may play an important role in the pathology of joint disease and inflammation by increasing the production of pro-inflammatory mediators produced by IL-1β.

The use of inhibitors to suppress NO production also generated novel data regarding p38 and ERK roles in the production of nitrite induced by IL-1β (chapter 8) in rat cartilage explants. It was concluded that these specific inhibitor molecules may be useful tools to elucidate specific signal transduction pathways involved in inflammation associated with inflammatory joint pathology.

It was the aim of this thesis to provide an insight into the nature of cartilage activity and cytokines within an *in vitro* system and that data from this study may provide an indication of the interactions between cartilage, fibroblasts and cytokines that may lead to cartilage breakdown in diseases such as rheumatoid arthritis.

Appendix I : Associated publications

Stephan, S., Purcell W.M., Punchard, N, and Chander C.L. (1999). Effects of granulocyte macrophage colony stimulating factor and interleukin-3 on prostaglandin-E2 and nitric oxide production in articular cartilage. *Mediators of Inflamm.* Vol. 8, suppl.1. S78.

Stephan, S., Purcell W.M. and Chander C.L. (1999). Granulocyte colony stimulating decreases glycosaminoglycans concentration and increases nitric oxide production in rat articular cartilage. *Inflam. Res.* 48, suppl 2, S126-127.

Stephan, S., Purcell, W. and Chander, C. (1999) Colony Stimulating Factors Regulate Nitric Oxide and Prostaglandin-E2 production by Rat Cartilage Chondrocytes. *International Journal of Tissue Reactions- Experimental and clinical aspects* 21, (4) 113-119.

Appendix II : Structure of IL-6

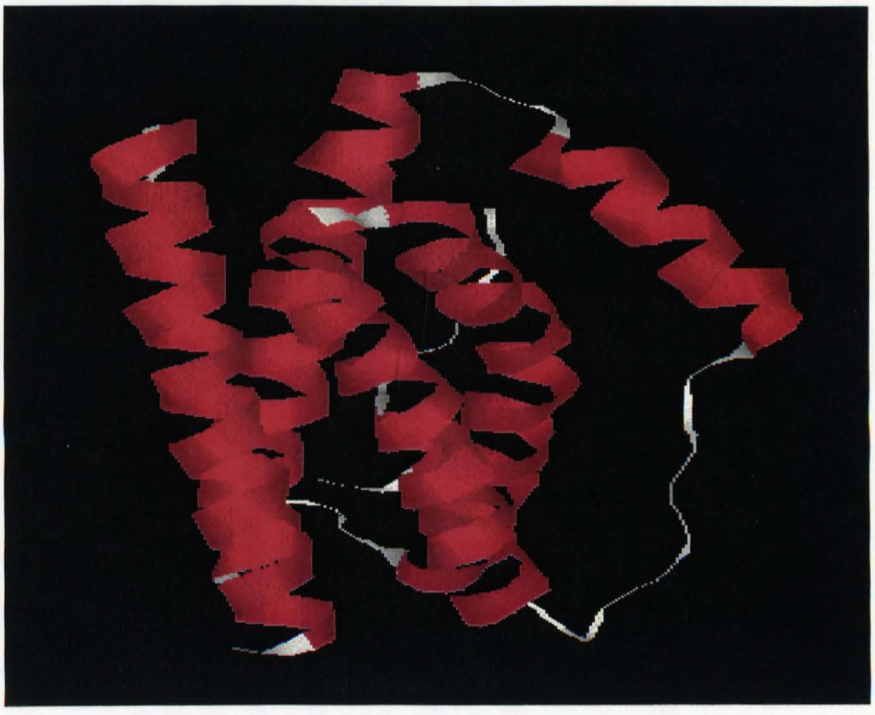
Image of IL-6 (Somers *et al.*, 1997) constructed from PDB files using RasMol.

(PDB identification no: 1ALU)

Appendix III : Structure of G-CSF

Image of G-CSF (, Zinc *et al.*, 1994) constructed from PDB file using RasMol.

(PDB identification no: 1GNC)



Appendix IV: Internet Web Page Addresses

Microanatomy Web Atlas (January 2001)

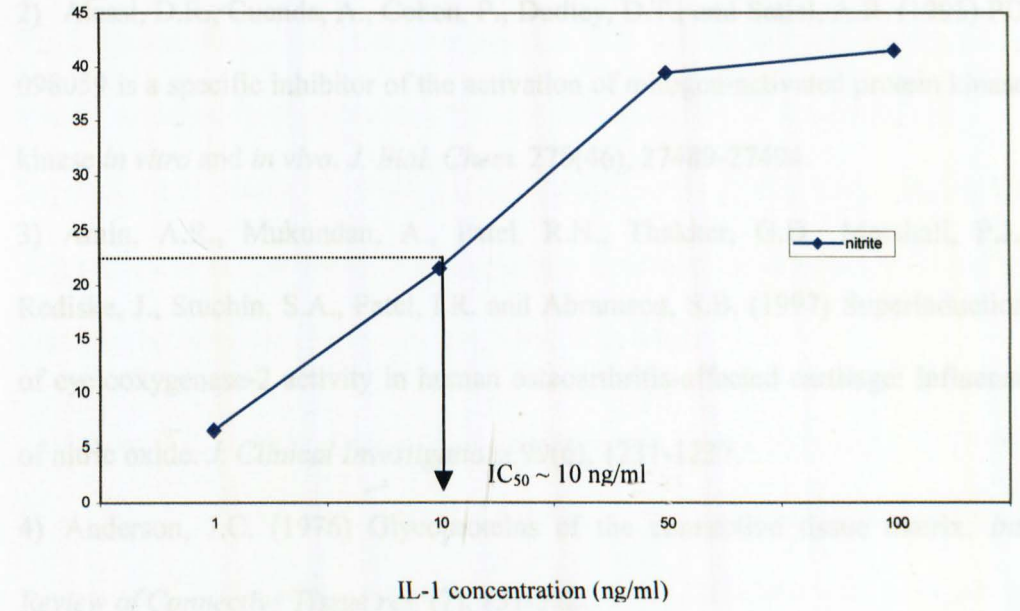
http://cellbio.utmb.edu/microanatomy/bone/cartilage_and_bone_cells.htm#CHONDROCYTES

American Academy of Orthopaedic Surgeons (January 2001)

http://orthoinfo.aaos.org/brochure/thr_report.cfm?Thread_ID=2&topcategory=Arthritis

Appendix V: IC_{50} of IL-1β

IC_{50} produced from IL-1β induced production of nitrite by rat cartilage explants during 3 days



Appendix V: Effect of IL-1β on nitrite production by rat femoral head cartilages during 3 days in culture. Control concentrations of nitrite were deducted from 1-100ng/ml treatments with IL-1β. The IC_{50} was calculated to be ~10 ng/ml.

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